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REGULATION OF HUMAN PARAINFLUENZA VIRUS TYPE 3 TRANSCRIPTION

A Thesis Submitted to the
School of Graduate Studies
University of Ottawa

In Partial Fulfillment of the
Requirements for the Degree of
Master of Science
Department of Microbiology and Immunology
School of Medicine

By

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ABSTRACT

To elucidate the roles of the junctional elements in HPIV3 transcription, cDNAs were constructed containing CAT and luciferase reporter genes flanked by sequences representing the HPIV3 termini necessary for transcription, replication and packaging. Sequences of the natural HPIV3 junctions were inserted between the reporter genes, and mutations were introduced into the gene end, intergenic and gene start sequences. RNA, transcribed *in vitro* from linearized plasmid DNA, was transfected into HPIV3-infected cells, and expression of reporter genes in the upstream and downstream positions was assayed. This approach allows the examination of the current model of paramyxovirus transcription, and documents the first analyses using bicistronic analogs to examine the regulatory roles of HPIV3 junctional elements in transcription.

Mutations to the gene end sequence abolished expression of the upstream and downstream genes. Deleting the gene start sequence at the junction resulted in increased expression of the upstream gene, but abrogated downstream gene activity. Alterations in the length of the intergenic trinucleotide resulted in decreased expression of both upstream and downstream genes. Mutations in the sequence of this nontranscribed trinucleotide resulted in decreased

activity of the upstream gene but no change in expression of the downstream gene.

The gene end sequence does not appear to contain the only signals for termination of transcription. The purine trinucleotide intergenic region is important for termination, but only the presence of three nucleotides appears to be necessary and sufficient for expression of the following gene. Results obtained from assaying reporter activity could often be interpreted in several ways. For example, the data could not distinguish between polymerase readthrough and premature termination. Two RNA detection methods were investigated and show promise as means for detecting and analyzing specific RNA species in transfected cells.

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The camaraderie of those in the department made time spent in the lab fun, while still providing the necessary learning environment. I chose well in coming here.

The most special thanks go to he who helped me start and he who helped me finish.

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ATP	adenosine triphosphate
ßGal	beta galactosidase
bp	base pairs
BPIV3	bovine parainfluenza virus 3
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
dATP	deoxyadenosine triphosphate
ddNTP	dideoxyribonucleotide
DI	defective interfering
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EtBr	ethidium bromide
F	fusion protein
FBS	fetal bovine serum
HPIV3	human parainfluenza virus type 3
HN	haemagglutinin-neuraminidase protein
L	polymerase protein
LB	Luria-Bertani
luc	luciferase
M	matrix protein
µL	microlitre
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid

NC	nucleocapsid
NDV	Newcastle disease virus
nm	nanometer
NP	nucleoprotein
nt	nucleotide
orf	open reading frame
pfu	plaque forming units
P	phosphoprotein
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	revolutions per minute
RSV	respiratory syncytial virus
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate buffer
STET	sucrose/tris/EDTA/triton-X buffer
TBE	tris/borate/EDTA buffer
TBS	tris buffered saline
TE	tris/EDTA buffer
TLC	thin layer chromatography
VSV	vesicular stomatitis virus
v	volume

I. Introduction

A. Overview

The negative strand RNA virus families include a number of clinically significant and economically important members. Negative sense RNA is not, on its own, infectious. Instead, it requires primary transcription by the viral RNA-dependent RNA polymerase. This requirement has been the major limitation to site-specific mutation of the genomic RNA of negative stranded viruses. The recent development of techniques allowing the *in vitro* manipulation of DNA representing RNA analogs of negative sense RNA virus genomes and rescue into infectious virus particles has resolved what had been called "one of the remaining technical limitations of recombinant DNA technology" (Pringle, 1991). Rescue technology permits defined genetic studies of negative sense RNA viruses and will aid the development of engineered vaccine strains. One application of these new techniques is the analysis of events surrounding transcription and replication of the negative sense RNA viruses. This thesis examines the control of transcription by *cis*-acting sequences of human parainfluenza virus type 3 (HPIV3).

B. *Paramyxoviridae*

HPIV3 is a member of the family *Paramyxoviridae*. The *Paramyxoviridae* are so named because they are similar (Para)

to the orthomyxoviruses such as influenza virus, in that they have an affinity for mucoprotein (myxo) receptors on erythrocytes. The paramyxoviruses have nonsegmented, negative sense genomes within helically symmetrical nucleocapsids encased in lipoprotein envelopes (Kingsbury, 1990). Virus particles are pleomorphic, ranging from 150-300 nm in diameter (Chanock and McIntosh, 1990). The *Paramyxoviridae* are thought to have evolved into a distinct family relatively recently, in evolutionary terms, as their host range is restricted to warm-blooded vertebrates. HPIV3 has been classified within the Paramyxovirus genus, which also contains the clinically important parainfluenza types 1, 2 and 4, as well as mumps virus. The *Paramyxoviridae* also include the Pneumovirus genus and the Morbillivirus genus, whose members include respiratory syncytial virus (RSV), and canine distemper virus (CDV) and measles virus, respectively (Kingsbury, 1990).

C. Human Parainfluenza Virus Type 3

HPIV3 is the most virulent of the four parainfluenza types (Sanchez *et al.*, 1986). This virus is an important pathogen of infants, young children and the immunocompromised. The primary infection rate of HPIV3 is 60-70% during the first thirty months of life (Bratt and Hightower, 1977). HPIV3 is often involved in lower respiratory tract infections such as croup, pneumonia and

bronchiolitis (Chanock and McIntosh, 1990). To date, there is no licensed vaccine or antiviral treatment for HPIV3 infections.

1. Proteins

The HPIV3 genome codes for at least seven proteins, the genes of which are grouped along the genome in the following order: core protein genes, envelope associated protein genes and the polymerase gene. The first, or most 3′ gene, codes for the nucleoprotein, NP, which associates tightly with viral genome and antigenome RNAs creating nuclease resistant structures. NP is the most abundant of the viral proteins synthesized.

The second gene encodes P, a phosphoprotein that is found as part of the viral core and is believed to be a part of the polymerase complex. In the *Rhabdoviridae*, another family of negative strand nonsegmented RNA viruses, the vesicular stomatitis virus (VSV) P protein has been shown to act as a transcriptional activator (Canter *et al.*, 1993), and is phoshorylated intracellularly by casein kinase II (Gupta *et al.*, 1995). Two N-terminal domains were recently identified in the P protein of Sendai virus, a related parainfluenza virus of mice. One domain appears important for RNA synthesis, and the other seems to help form a stable interaction with NP (Curran *et al.*, 1995).

The P gene of paramyxoviruses is multicistronic; overlapping open reading frames (orf) have also been observed in the P gene of rhabdoviruses (Spiropoulu and Nichol, 1993). HPIV3 belongs to a group of paramyxoviruses whose P orf spans the entire gene. The P genes of this group of viruses (which also includes Sendai virus, measles and CDV) contain overlapping P and C protein orfs. The C orf is in the N terminal region and positioned in the +1 frame relative to the P orf (Curran *et al.*, 1991). The C protein exhibits extensive amino acid identity between different viruses, but its functions remain unknown (Lamb and Paterson, 1991). For Sendai virus, the C orf also expresses multiple proteins (C', Y, Y') by ribosomal initiation from multiple start sites (Vidal *et al.*, 1990a). For HPIV3, mRNA synthesis off an additional initiation site has been observed *in vitro*; however, no evidence for this putative D protein has been demonstrated *in vivo* (Galinski *et al.*, 1986b).

The nontemplated addition of nucleotides (nt) to mRNAs is another phenomenon that permits the expression of additional polypeptides from the P gene. This cotranscriptional 'editing' involves the insertion of G residues by polymerase stuttering. Stuttering refers to the process whereby the polymerase pauses at a consensus site and slips upstream on the template a precise number of bases (Vidal *et al.*, 1990b; Kolakofsky *et al.*, 1991). A similar function for the viral polymerase has also been described for

the addition of A residues during polyadenylation. Indeed, the proposed frameshifting site for HPIV3 (3'-UUAAUUUUUUCCCCC-5') resembles the transcription termination sequence (see below)(Galinski *et al.*, 1992). For Sendai virus, measles, mumps and simian virus 5 (SV5), the viral polymerase stutters at the consensus sequence 3'-UUYUCCC-5' and inserts one or more G residues (Park and Krystal, 1992). The number of G residues inserted is virus specific (eg. 1G for Sendai virus and measles virus; 2Gs for SV5 and mumps virus) (Pelet *et al.*, 1991), and may depend on the stability of the nucleotide pair created by the displaced strand (Vidal *et al.*, 1990b). Insertion of one G occurs in 20-30% of Sendai P mRNA transcripts while insertion of two Gs occurs in approximately 5% of the mRNAs (Pelet *et al.*, 1991; Vidal *et al.*, 1990a). The resulting proteins are referred to as V and W, respectively, although they are underrepresented in virions (Curran *et al.*, 1991). For SV5 and mumps virus, it is the P protein that is derived from the frameshifted mRNA; unaltered mRNAs encode the V protein (Paterson and Lamb, 1990). The V protein of measles virus has been shown to bind zinc (Liston and Briedis, 1994), a characteristic feature of transcription regulatory proteins such as the adenovirus E1A protein. V proteins also have some amino acid homology with transcription regulators (Lamb and Paterson, 1991). The V protein is not believed to be expressed by HPIV3 (Galinski *et al.*, 1992).

M, the matrix protein, is encoded by the third HPIV3 gene. It is found on the inner surface of the viral envelope and associates with the nucleocapsids via ionic interactions (Galinski and Weschler, 1991). The M protein likely functions in gathering the viral nucleocapsids and glycoproteins at the cell surface before budding. The next genes found along the HPIV3 genome code for the integral HPIV3 envelope proteins, F and HN. F is the fusion protein; a glycoprotein that, in conjunction with HN, is responsible for virus-cell fusion events (Ebata *et al.*, 1991; Moscona and Peluso, 1992). The F protein undergoes post-translational proteolytic cleavage that is necessary for infectivity. Current antiviral research efforts target host proteases using aprotinin in aerosol form to suppress this cleavage (Orcharenko and Zhirnov, 1994). Haemagglutination and neuraminidase functions are encoded by the HN gene. The haemagglutinin specifically functions during attachment, while neuraminidase is believed to have a role in virus release (Galinski and Weschler, 1991).

The final, most 5′ gene, and by far the largest HPIV3 gene, encodes the polymerase, which is designated L. That L is expressed in the lowest levels during HPIV3 infection can be inferred from evidence from other virus systems, such as VSV (Meier, *et al.*, 1987). Along with P, L is believed to be responsible for catalyzing both transcription and replication (Moscona and Peluso, 1991). The locations of the HPIV3

proteins in the virion and the locations of the genes along the HPIV3 genome are depicted in Figure 1.

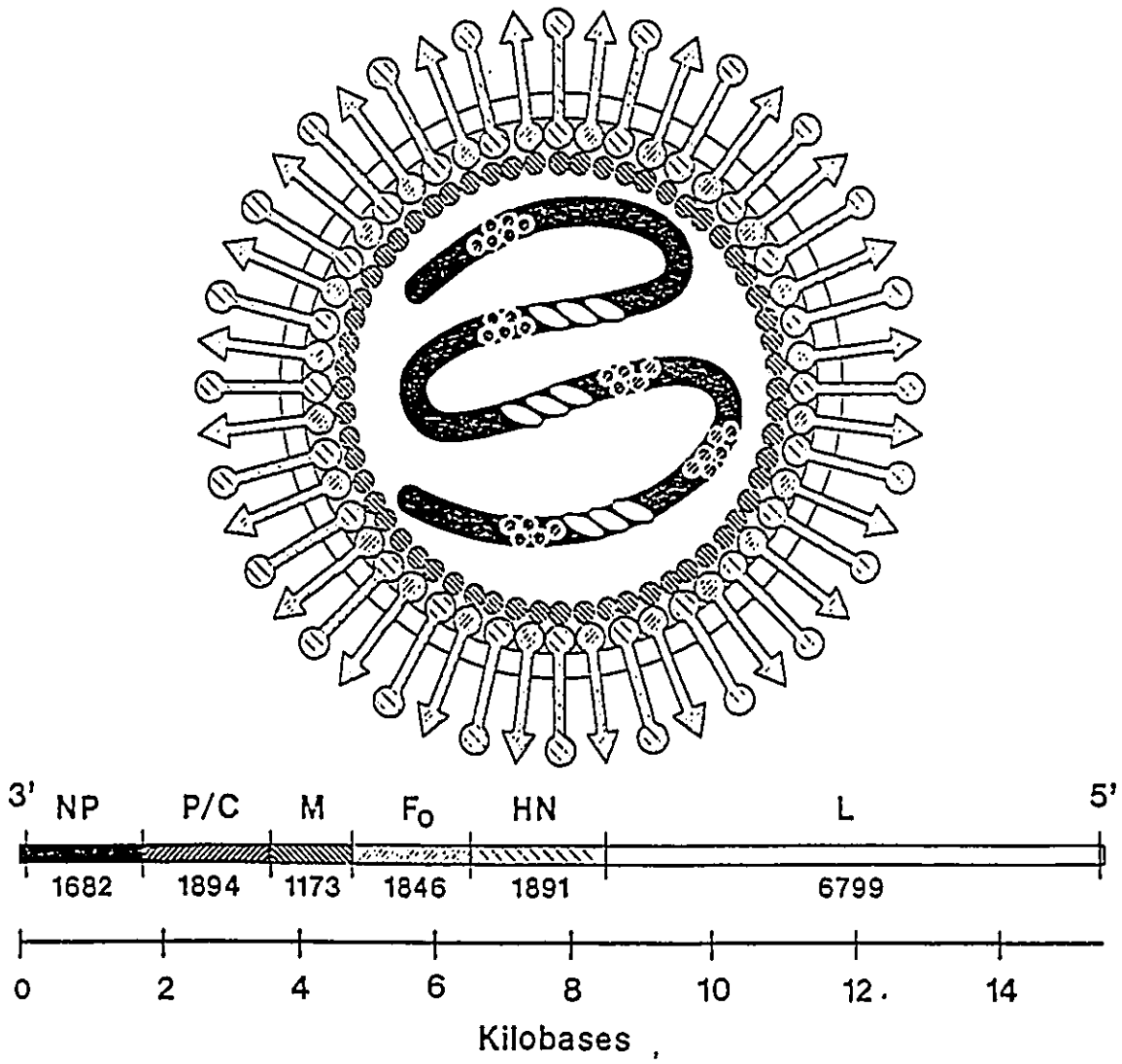
2. Genome Organization

HPIV3 is a negative sense RNA virus, which means that its genome is the complement of messenger RNA and is, therefore, noninfectious. The linear genome of HPIV3 has been sequenced in its entirety and is 15463 nt in length (Côté *et al.*, 1987; Dimock *et al.*, 1986; Elango *et al.*, 1986; Galinski *et al.*, 1986a, 1986b, 1987,1988; Jambou *et al.*, 1986; Prinoski *et al.*, 1987; Spriggs *et al.*, 1986). As intermolecular recombination has yet to be demonstrated for the nonsegmented negative stranded viruses, mutation appears to be the only way to generate variation (Pringle, 1991).

Genomic RNA is never found free; instead, it is associated with NP in a core structure which also includes the P and L proteins and is referred to as the nucleocapsid, or ribonucleoprotein (RNP). The nucleocapsid is the minimum unit of infectivity. Antigenome RNA is also encapsidated.

The 3′ end of the HPIV3 genome contains an extracistronic sequence that, by analogy with other nonsegmented negative strand viruses, has been designated as the template for the positive sense leader synthesis. Similarly, the 3′ end of the HPIV3 antigenome RNA contains sequences designated as the template for negative sense leader synthesis. 33 of the first 39 nucleotides at the 3′

Figure 1 **Schematic of HPIV3 virion and genome organization** (from Fields and Knipe, 1990). The viral components are labelled as follows: nucleoprotein (NP), phosphoprotein (P), polymerase-associated protein (C), matrix protein (M), haemagglutinin-neuraminidase (HN) and polymerase (L). Genes are shown in the order they appear along the genome. The number under each gene refers to the length (in bases) of the transcribed sequences of that gene.


3'
NP
P/C
M
F_o
HN
L
5'
1682
1894
1173
1846
1891
6799
0
2
4
6
8
10
12
14
Kilobases

and 5′ termini of the HPIV3 genome are complementary (Galinski *et al.*, 1988). The 8 terminal 3′ nucleotides of bovine parainfluenza virus type 3 (BPIV3), HPIV3, Sendai virus, Newcastle disease virus (NDV) and measles virus genomes are identical, indicating functional importance (Sakai *et al.*, 1987). The ability of HPIV1 or HPIV3 to replicate a Sendai virus defective-interfering (DI) RNA supports the hypothesis that *cis*-acting sequences at the viral genomic termini have been conserved (Curran and Kolakofsky, 1991). The conserved terminal sequences appear to have a role as transcription and replication 'promoters' in some viral systems; evidence will be examined for influenza virus in Section D.2.a. Another recent hypothesis suggests that the amount of complementarity in terminal sequences of VSV directly affects levels of replication or transcription (Wertz *et al.*, 1994).

3. Junctional Signals

At the junctions of the HPIV3 genes, there exist short consensus sequences of approximately 30 nt (Spriggs and Collins, 1986). These junctional sequences share homology with sequences identified in other negative strand RNA viruses, and, based on their location, have been divided into three regions: the gene end, the intergenic nontranscribed trinucleotide and the gene start. Conservation between each of the junctions emphasizes their biological importance. The

sequences of each of the natural junctions of HPIV3 appear in Table 1.

The gene end (3'-UUNAUNNUUUUU-5'; genome or vRNA sense) contains a U rich stretch that may be the signal and template for polyadenylation. Analogous sequences have been described for Sendai virus (3'-UNAUUCUUUUU-5') and VSV (3'-AUACUUUUUUU-5') (Gupta and Kingsbury, 1984). RSV appears to make use of two conserved gene end sequences: 3'-UCANUANAUUUU-5' and 3'-UCAAUNNNNUUUU-5' (Collins *et al.*, 1986). The gene end sequence for the M gene is slightly different from the other HPIV3 gene end sequences in that it contains a 8 nt insertion. Interestingly, an M-F readthrough transcript is uniquely abundant (Spriggs and Collins, 1986; Côté *et al.*, 1987), which may suggest that the extra 8 nt have a role in polymerase readthrough at this junction.

The intergenic trinucleotide of HPIV3 is 3'-GAA-5' (vRNA sense) in all junctions. This specific sequence has been observed in four of the five Sendai junctions (Gupta and Kingsbury, 1984) and all but one of the measles virus junctions (Crowley *et al.*, 1988). However, other paramyxoviruses, such as RSV and SV5, exhibit no conservation in the length or sequence of their intergenic regions (Spriggs and Collins, 1986; Hiebert *et al.*, 1985). Another nonsegmented negative sense RNA virus, VSV, has the consensus intergenic sequence 3'-GA-5'. The hypothesized role of the

Table 1 Putative transcription regulatory sequences of HPIV3 (vRNA sense).

Gene	Gene End	Intergenic	Gene Start
NP			UCCUAAUUUCU
NP/P	UAUUUAUUCUUUUU	GAA	UCCUAAUUUCU
P/M	UAUUUAUUCUUUUU	GAA	UCCUAAUUUCU
M/F	UAUUUAUUCUCUAUUAGUUUUU[1]	GAA	UCCUAAUUUCU
F/HN	UAUUAAUAUUUUUU	GAA	UCCUCAUUUCU
HN/L	UAUUUAUAUUUUUU	GAA	UCCUCGUUUCU
L	UUUCAUUCUUUUU		

[1]The underlined sequence demarcates the extra nucleotides of this junction.

intergenic trinucleotide according to the stop/start model of transcription (discussed below) is in termination.

The HPIV3 gene start has a consensus sequence of 3'-UCCUNNUUUCU-5' and may contain signals for resumption of transcription (Spriggs and Collins, 1986). The reported gene start sequence for Sendai virus is 3'-UCCCANUUUC-5', which has 80% sequence identity with the HPIV3 gene start (Gupta and Kingsbury, 1984). Conserved start sequences have also been identified for other paramyxoviruses, including: 3'-UGCCCAUCNUN-5' for NDV and 3'-CCCCGUUUA-5' for RSV (Pringle, 1991). The analogous sequence in VSV is 3'-UUGUC-5' (Banerjee and Barik, 1992).

Although three distinct junctional regions have been identified, transcriptional control undoubtedly involves a coordinated interaction of the three domains. As well, these elements need not be the only determinants to affect polymerase processivity.

D. Transcription and Replication

Negative sense RNA genomes have two template functions: transcription and replication. It is unknown how the switch between these two events is regulated. Much of what we know about the transcription and replication of HPIV3 has been inferred from other viral systems, including Sendai virus and VSV (for reviews see Banerjee, 1987; Banerjee and Barik, 1992). *In vitro* transcription systems have shown that

transcription acts independently of host constituents, although there have been reports for VSV (Banerjee, 1987) and RSV (Barik, 1992) that host cell extracts stimulate RNA synthesis. Contradictory reports suggest that the RNP complex **requires** cellular proteins for transcription (De *et al.*, 1990). ß-tubulin has specifically been identified as a positive transcription factor for Sendai virus and VSV (Moyer *et al.*, 1986), although ß-tubulin alone could not substitute for cell extract (Banerjee and Barik, 1992). Actin has also been shown to associate with HPIV3 RNP molecules, rendering them suitable for efficient transcription (De *et al.*, 1991; De *et al.*, 1993).

1. Transcription

1.a. Models of Transcription

There have been several models proposed for nonsegmented negative strand RNA virus transcription (Banerjee, 1987). The most widely accepted model of HPIV3 transcription is the stop/start model. This model postulates that the same polymerase molecule terminates and reinitiates at each junction. This model also proposes a single site of entry for the polymerase at the 3′ end of the genome, evidence for which has been put forth for VSV (Emerson, 1982) as well as several paramyxoviruses (Collins *et al.*, 1980; Glazier *et al.*, 1977; Dickens *et al.*, 1984). Attenuation of transcription (see Section 1.c., below) may therefore result

from failure of the polymerase to reinitiate consistently at downstream initiation sites.

A cleavage model has also been proposed for nonsegmented, negative stranded virus transcription (Banerjee *et al.*, 1977). In the cleavage model, RNA synthesis is initiated at the 3′ end of the genomic RNA, and cleavage occurs at specific sites along the growing chain. This model is generally not accepted due to the failure to demonstrate the presence of large uncleaved precursors. As well, it does not account for the nonequimolar mRNA synthesis observed without invoking differential mRNA stability.

A third model, the multiple initiation model, asserts that transcription is initiated at independent, internal sites, although the elongation of each mRNA is subject to prior transcription of the upstream gene. Such a model would require the polymerase to differentiate between 3′ replication and internal transcription initiation sites. The identification of a single, 3′ polymerase initiation site (Emerson, 1982) does not lend supportive evidence to this model.

1.b. Transcription Initiation

Ultraviolet transcriptional mapping techniques have indicated that the initiation site for transcription in paramyxoviruses is at the 3′ end of the genome (Collins *et al.*, 1980; Glazier *et al.*, 1977; Dickens *et al.*, 1984).

Primary transcription of HPIV3 is believed to be initiated by the synthesis of 3′ leader (Galinski and Weschler, 1991). The leader mRNA of HPIV3 is predicted to be a 52 nt, unpolyadenylated RNA synthesized in addition to the HPIV3 mRNAs. However, the presence of HPIV3 leader RNA has not yet been demonstrated. The leader may function in the switch from transcription to replication via antitermination (see Section D.2.)(Canter *et al.*, 1993), or in the control of encapsidation (Curran and Kolakofsky, 1991). However, the precise role of the leader remains unclear.

Initiation of transcription of individual influenza virus segments has been suggested to involve both the 3′ and 5′ terminal sequences that form a "panhandle" structure (Fodor *et al.*, 1994). It has been shown for influenza A that the RNA polymerase binds to the double stranded stem region of the panhandle (Yamanaka *et al.*, 1991), although the polymerase binds initially, and most tightly, to the 5′ terminus (Tiley *et al.*, 1994). Mutational analysis of the influenza virus promoter has revealed that the first 11 nt at the 3′ end constitute the minimum promoter, with nucleotides 9 to 11 being the most important (Seong and Brownlee, 1992b). Independent research has defined the polymerase recognition site to the 14 terminal nucleotides, with positions 2 and 11 being the most important (Piccone *et al.*, 1993). The polymerase does not seem to recognize a promoter if it is located internally (Li and Palese, 1992).

The nonsegmented negative stranded RNA viruses are not believed to make use of a panhandle in transcription initiation. Studies of VSV transcription *in vitro* have shown that the 3'-terminal 17 nt act as the optimal promoter, and that the 3'-terminal 3 nt (UGC) were essential for transcription (Smallwood and Moyer, 1993).

1.c. Transcription Attenuation

The number of each viral protein molecule associated with the viral genome has been determined for several negative stranded nonsegmented viruses, biochemically or using electron microscopy. The amount of each protein was seen to reflect the gene order. For Sendai virus, 2600 copies of NP, 300 copies of P/C and 40 copies of L have been reported to associate with each RNA genome (Calain and Roux, 1993). For VSV, the corresponding proteins are found in 2300, 230 and 50-60 copies per virion (Banerjee, 1987; Canter *et al.*, 1993). The relative amount of each of these proteins is mirrored by mRNA quantity. However, no evidence to date has documented any temporal control of transcription for these viruses. As rates of translation, as well as rates of mRNA degradation, are believed to be equivalent, the differences in mRNA abundance must be due to different rates of synthesis (Banerjee, 1987). Progressive attenuation of transcription with increasing distance from the promoter has been observed for VSV (Iverson and Rose, 1981). Attenuation

refers to the failure of the polymerase to reinitate at the downstream gene, resulting in nonequimolar synthesis of mRNAs. This attenuation has been localized to the junctions between genes, as the levels of mRNA synthesis for the 3′ and 5′ end of any given gene are equivalent (Iverson and Rose, 1981). Kinetic analyses indicate that significant pauses occur between transcription of neighbouring genes, perhaps due to processes that are slow compared to elongation, such as capping or polyadenylation (Banerjee, 1987). Pause times increase at each subsequent junction (Iverson and Rose, 1981), which may correlate with the increased failure to reinitiate. It has also been hypothesized for SV5 that increased pause times are inversely proportional to the production of readthrough transcripts (Hiebert *et al.*, 1985). The signals influencing polymerase pause times at specific junctions have not been determined.

Attenuation and the sequential nature of the transcriptive process are important regulatory features that balance the concentrations of viral proteins synthesized. One result is that the viral polymerase (L protein) is the least abundant protein in viruses and infected cells. It has been shown for VSV that low levels of L are important for efficient replication since only cells expressing low levels of the L protein could complement a temperature-sensitive L mutant (Meier *et al.*, 1987).

1.d. Polyadenylation versus Readthrough

Paramyxovirus mRNAs are predominantly monocistronic. The 5′ ends of the messages are capped and methylated (Barik, 1993), while the 3′ end is polyadenylated. Polyadenylation is thought to occur via transcriptional chattering at a U stretch in the gene end signal. Using information obtained from an internally deleted Sendai virus DI particle, it was observed that the viral RNA polymerase will not terminate unless it encounters specific nucleotides downstream of the first three Us of the polyadenylation signal (Hsu *et al.*, 1985). Data for influenza A virus (Luo *et al.*, 1991) suggest that a panhandle formed by complementary genome termini may have a role in polyadenylation and that the RNA polymerase stutters at a stretch of uridine residues as it hits the double stranded RNA barrier. Li and Palese (1994) have also described specific characteristics of this polyadenylation signal of segmented viruses. No evidence exists for such a structure in paramyxoviruses.

Polyadenylation and transcription termination in paramyxoviruses are coordinate events as evidenced by the lack of polyadenylate sequences between cistrons in Sendai virus readthrough transcripts (Gupta and Kingsbury, 1985). It has been estimated that 10% of the mRNAs synthesized during measles virus infection are polytranscripts (Crowley *et al.*, 1988). Readthrough transcription resulting in bicistronic messages occurs at an increased frequency at the

M/F junction in HPIV3 (Spriggs and Collins, 1986). It is not clear how or for what purpose this occurs, but it is of note that the sequence of the M/F junction is different from the others (Table 1). There has yet been no evidence to indicate whether polycistronic messages arise from abnormal events, are naturally occurring intermediates or have some unique function. For NDV, the presence of these mRNAs on polyribosomes seems to indicate that they do function as mRNAs (Wilde and Morrison, 1984); however, a specific role for polycistronic sequences has yet to be demonstrated.

2. Replication

Replication of all paramyxoviruses takes place in the host cytoplasm, independently of host DNA replication. Treatment of cells with actinomycin D, which prevents mRNA synthesis from DNA, indicates that viral synthesis occurs without concurrent host transcription (Barik, 1992).

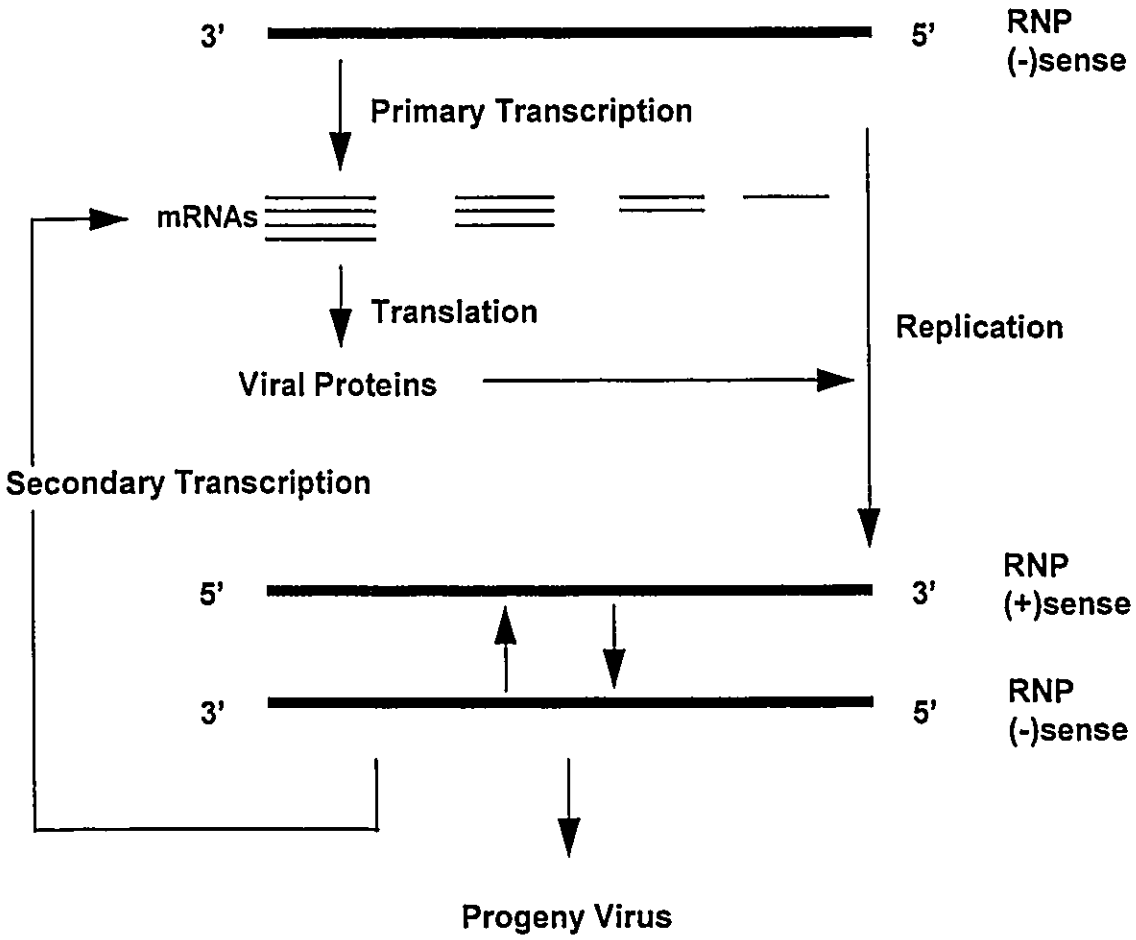
Replication must be preceded by primary transcription, which utilizes the virion associated RNA polymerase. Translation of the viral mRNAs yields the NP, P and L proteins necessary for genome replication. The negative sense genome is the template for the production of a complementary, antigenome strand. The antigenome is also found only as a NC structure, and is used as a template for the production of viral genomes. Transcription from these negative strand products is termed secondary transcription

and allows the amplification of viral macromolecular synthesis (Figure 2). Negative stranded RNA molecules are packaged into virions, which bud from the host cells (Banerjee and Barik, 1992).

Not much has yet been learned about the specific signals controlling replication of negative sense virus genomes. Some recent evidence may indicate that the amount of complementarity in the terminal sequences may direct replication or transcription. For VSV, (Wertz *et al.*, 1994) the natural termini result in relatively low levels of replication but high levels of transcription in infected cells. RNAs with trailer sequences (or alternatively leader sequences) at both ends showed increased replication and decreased transcription. Thus, it appears that the extent of complementarity is an important determinant of whether replication or transcription predominates. This observation may help explain the replicative dominance of DI genomes, such as copyback DIs, which have identical termini (Wertz et al., 1994).

A role for the nucleoprotein in controlling the switch between replication and transcription has been suggested. The "minimal" model, proposed for VSV (Blumberg *et al.*, 1981), portrays NP as an antiterminator that binds to the nascent leader RNA and masks the termination signals (Moyer and Horikami, 1991). Concurrent assembly of NP with the emerging chain seems to be required for elongation as well as

Figure 2 Summary of the HPIV3 replication strategy (adapted from Fields and Knipe, 1990). Primary transcription of the RNP yields mRNAs that are translated into the viral proteins necessary for genome replication. Replication results in an abundance of templates for secondary transcription and production of progeny virus particles via a positive sense intermediate.


3'
5'
RNP
(-)sense
Primary Transcription
mRNAs
Translation
Viral Proteins
Replication
Secondary Transcription
5'
3'
RNP
(+)sense
3'
5'
RNP
(-)sense
Progeny Virus

junctional readthrough (Vidal and Kolakofsky, 1989; Curran and Kolakofsky, 1991). In this model, transcription is envisioned to proceed until levels of NP become high enough to block termination, thus allowing replication of the entire RNA molecule. Some have added to this model by suggesting that the phosphoprotein regulates replication by controlling nucleoprotein availability. Indeed, in the VSV system, the molar ratio of N/P (where N is analogous to the HPIV3 NP protein) was observed to be critical for optimum replication (Banerjee, 1987). This model still does not explain how leader encapsidation modifies the polymerase to suppress intergenic signals for transcription (Banerjee and Barik, 1992). A model for replication must also address how the 3' ends of both genome and antigenome RNA molecules act as replication origins, whereas only the 3' end of the negative sense RNA acts as a transcription initiation site.

3. Obstacles to Progress in Genetic Analyses

The models of transcription and replication for HPIV3, and indeed, for most negative stranded viruses, are very sketchy. As well, there has been no molecular analysis of the elements involved. To summarize, the major hurdle to understanding what is occurring in this system is that the noninfectious genome does not allow *in vivo* analysis of constructed mutations. As well, there is no evidence of homologous recombination in nonsegmented negative stranded

viruses. Because the genome is not segmented, reassortment, an often exploited technique for the introduction of mutated genes into segmented viruses such as influenza virus, does not occur (Bergmann *et al.*, 1992). The large size of nonsegmented virus RNA genomes has also made cloning of full length genomes difficult. Furthermore, no encapsidation systems exist that would allow *in vitro* analysis of large genome analogs of the nonsegmented RNA viruses. These hurdles limit the analysis of viral functions as well as applications such as vaccine production. "A full analysis awaits technology which will enable genetic information manipulated *in vitro* to be rescued into infectious virus" (Pringle, 1991).

E. Rescue

Because of the difficulties with the manipulation of negative stranded viral genomes, the elucidation of many viral processes and their regulation has been slow. Prior to 1990, work was done using *in vitro* systems with cell extracts. These systems were limited in their ability to directly examine the viral genetic material. This limitation has recently been overcome by the ability to "rescue" synthetic RNAs. Much of the current rescue work has been reviewed recently (Garcia-Sastre and Palese, 1993). In this thesis, the term rescue refers to the transcription and

replication of synthetic analogs of viral genomes, as well as their packaging into progeny virus particles.

1. Development of Rescue Strategies

1.a. Segmented Viruses

Reconstitution of influenza A virus RNA with NP and polymerase proteins into a functional RNP complex was achieved in 1989 (Parvin et al., 1989). Luytjes *et al.* (1989) then used reconstituted RNP particles to rescue a synthetic analog of influenza A virus NS segment into infectious particles. Wild type, helper influenza virus provided viral proteins necessary for the production of progeny. One immediate prospect was the possibility of introducing specific mutations into influenza genes (Enami *et al.*, 1990). The influenza transfection protocol was soon improved 100 fold by coupling RNP formation to *in vitro* transcription of RNA analogs (Enami and Palese, 1991).

Several alternative rescue protocols have been developed for influenza viruses. Yamanaka *et al.* (1991) used RNPs from helper viruses, instead of intact virus, to drive replication of the reconstituted RNPs. Also, micrococcal nuclease treated cores have been reconstituted with synthetic RNAs (Seong and Brownlee, 1992a). A helper-independent rescue system, developed by Huang *et al.* (1990), provides the necessary viral proteins to support rescue with transfected plasmids containing cDNA copies of viral genes under the

control of the T7 RNA polymerase promoter. Expression of viral genes in cells is driven by T7 RNA polymerase made by a vaccinia virus engineered to express this polymerase (Fuerst *et al.*, 1986). Recently, a cell line has been established that expresses NP and the influenza polymerase proteins in response to dexamethasone (Kimura *et al.*, 1992).

These rescue systems have allowed the study of the molecular mechanisms of influenza transcription and replication. Specifically, these techniques permitted characterization of the polyadenylation signal (Li and Palese, 1994), promoter sequences (Piccone *et al.*, 1993), the neuraminidase gene (Liu and Air, 1993), replication proteins (Huang *et al.*, 1990), mechanisms of attenuation (Muster *et al.*, 1991; Luo *et al.*, 1992), and actions of antivirals (Huang *et al.*, 1992). Foreign proteins have been expressed as part of one of the viral segments, either as a fusion protein containing a protease signal (Percy *et al.*, 1994) or by the creation of a functionally bicistronic influenza gene segment (Garcia-Sastre *et al.*, 1994). Recently, specific mutations have also been introduced into the HA gene of influenza B virus (Barclay and Palese, 1995).

1.b. Nonsegmented Viruses

Advances in rescue technology for the nonsegmented viruses have lagged behind those made for influenza A, basically because of the larger genome size. De *et al.*

(1990) synthesized HPIV3 mRNA *in vitro* with cell extracts. Rescue for a non-segmented virus was achieved in 1991 for Sendai virus (Park *et al.*, 1991). This system used wild-type helper virus to direct replication of the synthetic analog. Synthetic analogs of RSV were rescued in the same year by Collins *et al.* (1991). In 1993, a similar system was successfully used to rescue HPIV3 genome analogs (Dimock and Collins, 1993; De and Banerjee, 1993).

A different approach was developed for VSV (Pattnaik and Wertz, 1990). This group cloned cDNA copies of the viral genes and of a DI genome into plasmids under the control of the T7 promoter. These plasmids were transfected into cells that had been infected with vaccinia virus expressing T7 polymerase, and were able to direct replication of a VSV DI particle. It was seen that replication and amplification of the VSV DI particles required only the N, P, and L proteins. Rescued RNA was detected directly via Northern analysis. Cloned Sendai virus DI genomes have also been rescued in this fashion (Calain *et al.*, 1992). It has been shown that measles virus nucleocapsid protein can function in the replication of Sendai DIs (Chandrika *et al.*, 1995).

Rescue systems using cloned copies of the genes for viral core proteins do not look at packaging. However, Pattnaik and Wertz (1991) have reported that cells expressing the VSV M and G proteins, as well as N, P and L, could support replication, assembly and budding of the DI

particles. DI particles are transcriptionally inactive, so this system has its limitations. It was noticed that a precise 3′ terminus of the minigenome was critical for success (Pattnaik *et al.*, 1992), a feature common in other rescue systems. Recently, this system was used to show that the 36 - 5′ terminal and 51 - 3′ terminal nucleotides are sufficient for the encapsidation, replication and packaging of VSV minigenomes (Pattnaik *et al.*, 1995).

Most recently, rescue of synthetic analogs of rabies virus has been reported (Conzelmann and Schnell, 1994). The rabies system is similar to that developed for VSV in that the essential viral proteins are encoded on plasmids with transcription driven by vaccinia virus expressing T7 polymerase. This system is also the first to report rescue of a full length genome analog entirely from cloned cDNA (Schnell *et al.*, 1994). Approximately 50% of the RSV genome has been rescued successfully (Collins *et al.*, 1993).

As for influenza virus, rescue techniques for the nonsegmented viruses have allowed analyses of many viral structures and processes. Rescue techniques have furthered the examination of nontemplated nucleotide addition in P gene transcription (Park and Krystal, 1992), as well as the role of terminal complementarity in replication (Wertz *et al.*, 1994). Heterologous G proteins have been seen to replace the rabies glycoprotein in all phases of the virus life cycle (Mebatsion *et al.*, 1995).

2. The Rule of Six

As previously mentioned, the role of the nucleocapsid protein is to surround and stabilize (ie. encapsidate) the genome and antigenome molecules. Stoichiometric data for Sendai virus indicate that each NP protein molecule specifically contacts six nucleotides, creating a nuclease resistant structure (Egelman *et al.*, 1989). Calain and Roux (1993) reported that only Sendai DI genomes that have a genome whose number of nucleotides is evenly divisible by six can be rescued. They contend that uneven nucleotide-nucleoprotein associations leave free nucleotides at the genomic termini and do not "serve as a proper replication promoter" (Calain and Roux, 1993). The rule of six does not preclude the requirement for specific signal sequences at the viral RNA termini, but may affect successful replication of genomes or genome analogs.

II. Objectives

The sequences at the gene junctions in the HPIV3 genome are highly conserved. It is believed that these gene junctions contain all the necessary signals for controlling transcriptional events. A rescue system for HPIV3 has been developed in this laboratory (Dimock and Collins, 1993) that results in the transcription and replication, in infected cells, of genome analogs of HPIV3 synthesized *in vitro* from cDNA. The main objective of this project was to use this HPIV3 rescue system to analyze viral gene junctions believed to be important for polymerase termination, polyadenylation and polymerase reinitiation during transcription.

Specific objectives for this thesis were:

1. to synthesize bicistronic HPIV3 genome analogs containing two reporter genes (CAT and luciferase (luc)).

2. to utilize the bicistronic constructs to examine the roles of the gene end, intergenic and gene start sequences in transcription.

3. to examine the extent to which HPIV3 is governed by the 'Rule of Six'.

III. METHODS AND MATERIALS

A. Cell Culture and Virology

1. Cells

The Rhesus monkey kidney cell line, LLC-MK2, was obtained from the American Type Culture Collection. The human embryonic kidney cell line, 293, and the human epidermal carcinoma cell line, HEp-2, were kindly provided by Dr. P. Collins (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). All cell lines were grown in autoclavable minimal essential medium (MEM; Gibco/BRL) with 5% (LLC-MK2) or 10% (HEp-2 and 293) fetal bovine serum (FBS; Gibco/BRL) that had been heat inactivated at 56°C for one hour. Media were supplemented with 2 mM L-glutamine, 0.23% (w/v) sodium bicarbonate and 50 μg/mL gentamycin sulphate. Cell monolayers were maintained in 100 mm diameter polystyrene dishes (Corning) and grown at 37°C in a 5% CO_2 atmosphere in a Shellab incubator (Sheldon Manufacturing Inc.).

LLC-MK2 and HEp-2 cells were split weekly at a ratio of 1:10 while 293 cells were split twice weekly at a 1:3 ratio. Passaging was performed on confluent monolayers by first washing twice with 5 mL of prewarmed Tris-buffered saline (TBS; 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.6 mM glucose, 25 mM Tris-Cl pH 7.2). 0.005% trypsin/0.002% EDTA in 2 mL TBS were added, and plates were incubated at 37°C for 2-3

minutes. The trypsin was inactivated by the addition of culture medium, and cells were diluted into fresh culture plates.

2. Virus

A stock of human parainfluenza virus type 3 (HPIV3) strain WASH/47885/57, propagated in HEp-2 cells, was obtained from Dr. P. Collins (NIAID, NIH).

3. Preparation of Virus Stocks

Confluent plates of LLC-MK2 or HEp-2 cells were infected at a multiplicity of infection (MOI) of 0.1 in serum-free medium. Incubation at 37°C for one hour with occasional tilting allowed virus to adsorb to the cells. The inoculum was replaced with 7 mL fresh MEM containing serum, and plates were incubated for 72 hours. After incubation, supernatants were pooled and centrifuged for 5 minutes at 1000 rpm in a Sorvall GLC-2B centrifuge (Dupont) to remove debris. HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid), pH 7.5 was added to a final concentration of 50 mM, and the supernatant was aliquoted into glass vials and stored at -80°C.

4. Plaque Assay

Confluent monolayers of HEp-2 cells in 6 well plates (Corning) were used for plaque assays. Serial dilutions of

the virus stock were prepared in 0.5 mL serum-free MEM. Cells were infected for 1 hour at 37°C. 2.5 mL of 0.8% agarose/MEM were overlayed and incubation continued until visible plaques were seen (4d). 2 mL of a formalin (10% (v/v) of 37.5% formaldehyde), 0.8% (w/v) NaCl solution were added to each well. After one hour at 37°C, the agarose overlays were washed off, and monolayers were stained for thirty minutes with 2 mL 0.1% (w/v) crystal violet.

B. Restriction and Modification of DNA

1. Enzymes

Restriction endonucleases and modifying enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, Promega Biotech and Pharmacia Canada.

2. Restriction Digests

All DNA restriction digests were done in the buffers obtained from the suppliers, at the recommended temperatures. Digestions were stopped by heat inactivation where possible.

3. Cloning of Annealed Oligonucleotides

Oligonucleotides used in the construction of mutants by direct cloning were kindly provided by Dr. P.L. Collins (NIAID, NIH) (see Appendix). Complementary oligonucleotides were annealed by heating 1 μg of each DNA pair in 10 μL H_2O

to 95°C for 5 minutes and then allowing the sample to cool slowly.

Ligation of DNA was performed in 20 μL reactions with 500 ng pPIV-CL (see Figure 4) or pPIV-LC (see Figure 5) that had been digested with *Sac*II and *Bam*HI and 1 μg of the oligonucleotide pair. Reactions were performed in 1 mM ATP, 50 mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 1 mM DTT and 5% (w/v) polyethylene glycol-8000, using 2 units T4 DNA ligase. Ligations were allowed to incubate overnight at 12°C.

E. coli DH5α F′ cells were transformed with ligated DNA as described (Section III.C). Positive clones were selected by the lack of digestion of plasmid DNA with *Hpa*1, and plasmid DNAs from potential mutants were sequenced (Section III.E) to verify the mutations.

4. Site-Directed Mutagenesis

The Kunkel method of mutagenesis was used (Kunkel, 1985; Kunkel *et al.*, 1987) with the aid of a Muta-Gene Phagemid *In Vitro* Mutagenesis kit (Biorad). *E. coli* CJ236 were electrotransformed (Section III.C.2) with the phagemid pCAT-lucf1 or pluc-CATf1. The phagemids were prepared by Dr. K. Dimock by insertion of the *Xmn*I/*Sac*I fragment of pBluescript KSII (Stratagene) containing the f1 origin of replication into pPIV-CL-NP/P and pPIV-LC-NP/P. 50 mL cultures were grown in 2YT (1% yeast, 1.6% tryptone, 0.5% NaCl) at 37°C, with agitation at 225 rpm, to an absorbance of 0.3 at 600 nm.

Cultures were infected with 20 pfu/cell M13KO7. After one hour at 37°C, kanamycin was added to a concentration of 70 μg/mL. Incubation continued overnight at 37°C. 30 mL of the overnight culture were pelleted in a Beckman model J2-21M centrifuge at ~17,400 x g at 4°C for 15 minutes. The supernatant was transferred to a fresh tube, and 150 μg RNAse A were added. After a 30 minute incubation at room temperature, 7.5 mLs of 3.5 M ammonium acetate/20% polyethylene glycol-6000 were added, and the tubes were incubated on ice for 30 minutes. Phage particles were collected by centrifugation at ~17,400 x g for 15 minutes. The resulting pellet was resuspended in 400 μL of high salt buffer (300 mM NaCl, 100 mM Tris, pH 8.0, 1 mM EDTA). Samples were transferred to microfuge tubes and incubated on ice for thirty minutes.

Single stranded, uracil containing DNA was extracted from the phage particles by extracting twice with phenol, once with phenol/chloroform-isoamyl alcohol (25:24:1) and three times with chloroform-isoamyl alcohol (24:1). DNA was precipitated with 1/10 volume 7.8 M ammonium acetate and 2.5 volumes EtOH.

200 pmol of each mutagenic oligonucleotide (see Appendix) was phosphorylated in a 30 μL reaction (100 mM Tris, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 0.4 mM ATP) containing 4.5 units T4 polynucleotide kinase (Biorad). Reactions proceeded for 45 minutes at 37°C and were stopped by heating

at 65°C for 10 minutes. Phosphorylated oligonucleotides were diluted to 6 pmol/μL with TE.

Phosphorylated oligonucleotides and uracil-containing single stranded DNA were annealed in a 10:1 molar ratio in a 10 μL reaction (20 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 50 mM NaCl). A negative control was prepared that did not contain oligonucleotide. Samples were heated to 70°C and allowed to cool at 1°C/min to 30°C. Complementary strand synthesis and ligation were achieved by the addition of 0.5 units T7 DNA polymerase, 3 units T4 DNA ligase (Biorad) and synthesis buffer (final concentration: 0.4 mM each dNTP, 0.75 mM ATP, 17.5 mM Tris-HCl pH 7.4, 3.75 mM $MgCl_2$, 0.5 mM DTT). Samples were incubated 5 minutes on ice, 5 minutes at 25°C and 90 minutes at 37°C. An aliquot of this reaction was used to transform electrocompetent *E. coli* DH5α F' cells as described below. Mutant confirmation was done by DNA sequence analysis (Section III.E).

C. Electro-Transformation

1. Preparation of electrocompetent cells

1L LB broth was inoculated with 1/100 volume of a fresh overnight culture of *E. coli* DH5α F'. Cells were grown with agitation (225 rpm) to an OD_{600} of 0.5-1.0. The culture was chilled on ice for 30 minutes, and cells were centrifuged in a Beckman JA-10 rotor at ~1600 x g for 10 minutes at 4°C. The pellet was resuspended in 1 L cold, sterile water, and

cells were collected by centrifugation. The resulting pellet was resuspended in 500 mL cold water and centrifugation was repeated. The pellet was resuspended in 20 mL of cold 15% glycerol. Cells were pelleted by centrifugation in a JA-20 rotor at 1100 x g for 10 minutes at 4°C. Final resuspension was in 3 mL 15% glycerol. Aliquots of cells were frozen on dry ice and stored at -80°C.

2. Electroporation

1 - 10 ng of DNA in a volume of 1 μL was mixed with 20 μL electrocompetent cells and placed in a sterile cuvette in the electroporation chamber (Gibco/BRL). Conditions for electroporation were as follows: resistance=4 kΩ, capacitance =330 μF, resistance of media=low setting. After electroporation, cells were incubated at 37°C for 1 hour in 1 mL SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl supplemented with 10 mM $MgSO_4$ and 20 mM glucose). A 100 μL aliquot of the transformed cells was plated on LB agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 50 μg/mL ampicillin (Boehringer Mannheim). Colonies were allowed to grow overnight at 37°C.

D. DNA Isolation and Analysis

1. Small scale isolation of plasmid DNA (miniprep)

The miniprep DNA isolation procedure used was a boiling method (Holmes and Quigley, 1981). Single colonies were used

to inoculate 5 mL cultures of LB broth containing ampicillin, which were incubated overnight at 37°C. 1.5 mL of the culture were transferred to a microfuge tube, and cells were pelleted. The pellet was resuspended in 500 μL STET (8% sucrose, 10 mM Tris-HCl pH 8.0, 50 mM EDTA, 0.5% Triton X100). 30 μL 10 mg/mL lysozyme (Sigma, St. Louis, MO) in 10 mM Tris-Cl pH 8.0 were added, and tubes were mixed by inversion for one minute. Tubes were placed in a boiling water bath for 40 seconds and then centrifuged for 15 minutes at room temperature. The clot was removed with a sterile toothpick. 1 μL 10 mg/mL RNAse A (Boehringer Mannheim) was added, and tubes were incubated for 5 minutes at room temperature. After an extraction with 500 μL chloroform-isoamyl alcohol (24:1), DNA was precipitated by the addition of NaCl to a final concentration of 150 mM and 1 mL isopropanol. Pelleted DNA was washed once with 70% EtOH, and samples were resuspended in 50 μL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). 5 μL were examined on an agarose gel as described in Section III.D.3.

2. Large scale isolation of plasmid DNA (maxiprep)

Large scale DNA preparations were performed using Qiagen reagents and columns. DNA was resuspended in TE to approximately 1 μg/μL, according to the column yield. The exact concentration was determined by spectrophotometry.

3. Agarose gel electrophoresis

DNA electrophoresis was performed using 1% agarose gels in Tris-borate buffer (TBE; 89 mM Tris, 89 mM boric acid, 2 mM EDTA pH 8.0). Samples were prepared by adding 1/10 volume load buffer (50% glycerol, 0.1 M EDTA, 1% SDS, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Gels were run in a Horizon 58 (Gibco/BRL) apparatus at approximately 10 volts/cm. Gels were stained in 1 μg/mL ethidium bromide (EtBr) and destained briefly in H_20. Photography was performed using a Polaroid MP4 land camera equipped with a transilluminator (BIO/CAN Scientific).

E. Sequence Analysis

1. Dideoxynucleotide sequencing

The dideoxynucleotide chain termination method of sequencing (Sanger *et al.*, 1977) was employed exclusively throughout this project. To sequence across the gene junctions in CAT-Luc constructs, primers 3062 and 4516 were used (see Appendix). Primers 4492 and 3061 were used to sequence across the Luc-CAT junctions in both directions. 8 μL DNA from a miniprep were denatured using 2 μL 2 N NaOH for 8 minutes. 50 ng of the appropriate oligonucleotide primer, 3 μL 3 M NaOAc pH 5.5 and 75 μL 100% EtOH were added to the denatured DNA. Samples were placed at -80°C for 20 minutes,

then centrifuged at 12,000 rpm for 20 minutes at 4°C. Pellets were washed with 100% EtOH and dried.

For the labelling reaction, 11 μL water, 2 μL 5x Sequenase™ buffer (40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl), 1 μL 0.1M DTT, 0.4 μL labelling mix, 0.5 μL S^{35} dATP (Du Pont, Mississuaga, ON) and 2 μL Sequenase™ (United States Biochemicals, Cleveland, OH), diluted 1/8, were added to the DNA-primer pellet. 2.5 μL of termination mixes were added to each of four tubes and warmed to 37°C. 3.5 μL of the labelling reaction were added to each of the four tubes. Tubes were incubated at 37°C for 10 minutes, and the reaction was stopped with 4 μL stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Samples were stored at -20°C for up to one week.

2. Sequencing gels

0.4 mm thick, 6% polyacrylamide gels were used. 5.7 g acrylamide, 0.3 g bis-acrylamide, 42.0 g urea, and 20 mL 5x TBE were mixed and the volume was adjusted to 100 mL with water. After the solution was filtered (0.45 μm filter), 450 μL 10% ammonium persulfate and 45 μL TEMED were added and the gel was poured immediately. Gels were allowed to polymerize for 2-16 hours. Before loading samples, the gel was electrophoresed 1 hour at 56 W in TBE buffer on an IBI model STS-45 sequencing apparatus. Samples were heated to 70°C for two minutes, and 3 μL were loaded onto the gel.

Electrophoresis was continued for 2 hours at 56 W. After electrophoresis, gels were lifted onto Whatmann 3MM paper and covered with plastic wrap. Gels were dried in a Biorad model 583 gel drier and exposed to Cronex X-ray film (Dupont) for two days.

F. *In vitro* Transcription

In vitro transcription reactions were performed according to protocols recommended by the supplier (Promega) with some modifications. Each 100 μL reaction contained 20 μL 5X transcription buffer, 10 mM DTT, 100 units RNAsin and 1 mM each dNTP, heated to 37°C. 2 μg DNA and 60 units T7 RNA polymerase were added. Reactions proceeded at 37°C for 3 hours. The amount of RNA synthesized was estimated by running 3 μL of the reaction on an agarose gel (Section III.D.3). Samples were stored at -80°C.

G. Transfection

293 cells were split 1:3 into 6 well dishes and incubated at 37°C until they had reached confluency. The medium was removed, and cells were infected with 1 mL HPIV3 (10^7 pfu/mL; MOI=5-10). Cultures were incubated for 1 hour at 37°C. The viral inoculum was removed, wells were washed with 1.5 mL serum-free MEM, and 1 mL of the appropriate transfection mixture was added. Transfection mixes were prepared in 1 mL OPTIMEM (Gibco/BRL) in polystyrene tubes

(Sarstedt). The mixes contained 12 μL Lipofectace (Gibco/BRL) and 20 μL of an *in vitro* transcription reaction. Cells were transfected for 3 hours at 37°C. The transfection mix was replaced with 1.5 mL MEM containing 10% FBS, and plates were incubated 24 hours at 37°C. A summary of this rescue protocol appears in Figure 3.

H. Assays

1. CAT Assay

Cells from individual wells were harvested by pipetting up and down and transferred to 1.5 mL microfuge tubes. Cells were pelleted for 1 minute at full speed in a microcentrifuge at room temperature. Supernatants were transferred to fresh microfuge tubes and frozen at -80°C, or discarded. The cells were washed in 500 μL CAT wash buffer (150 mM NaCl, 40 mM Tris-HCl pH 7.5, 1 mM EDTA) and pelleted again. Cells were resuspended in 60 μL cold 250 mM Tris-HCl pH 7.5 and subjected to three rounds of freezing (liquid N_2) and thawing (37°C). Cellular debris was pelleted 5 minutes at 12k rpm at 4°C. The cell lysate was transferred to a fresh tube and flash frozen. Long term storage was at -80°C.

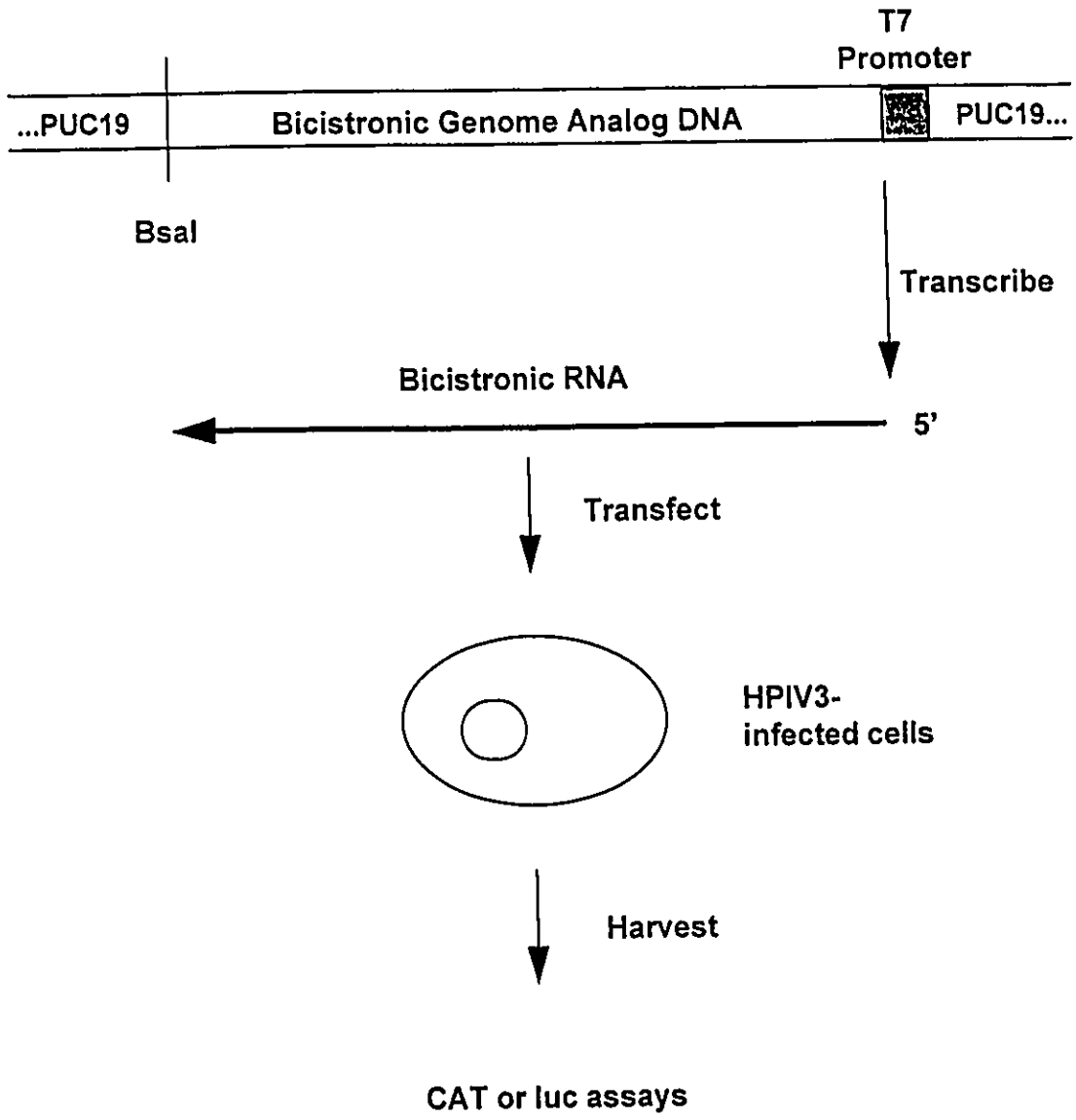
100 μL of a CAT Mix (250 mM Tris-HCl pH 7.5, 4 mM acetyl coenzyme A (Boehringer Mannheim), 0.05 μCi D-threo [dichloroacetyl-1-^{14}C] chloramphenicol (Amersham)), were added to 50 μL of cell lysate. Tubes were incubated 6 hours at 37°C.

1 mL ethyl acetate was added to each sample, and samples were vortexed and centrifuged for 3 minutes at 12k rpm at room temperature. 900 μL of the upper, organic phase was transferred to a fresh tube. Samples were dried in a Speed-Vac concentrator (Savant). Pellets were resuspended in 8 μL ethyl acetate. Samples were spotted on a silica thin layer chromatography (TLC) plate (Baker) and air dried briefly before developing in a 95:5 (v/v) chloroform/methanol environment. The TLC plates were dried, sprayed with EnHance (DUPONT) and exposed to X-ray film at -80°C for several days. For quantitation, radioactive spots on the TLC plate corresponding to acetylated and nonacetylated forms of chloramphenicol were cut out and counted in a liquid scintillation counter (LKB) using 3 mL EcoLite scintillation cocktail (ICN Biomedicals). Percent conversion for each sample was determined by dividing the acetylated counts by the total counts.

2. Luciferase Assay

Cloned firefly luciferase can be expressed from animal cells; it catalyses a luminescent reaction with luciferin, ATP and O_2 (Wood and DeLuca, 1987). The luciferase assays were performed with reagents provided in a luciferase assay kit (Promega). 10 μL of cell extracts, prepared as described above for CAT assays, were mixed with 100 μL Luciferase Assay Reagent (Promega) that had been equilibrated to room

Figure 3 Schematic of the HPIV3 rescue protocol. DNA is linearized with *Bsa*I and transcribed *in vitro*. RNA is transfected into 293 cells that have been infected with wild-type HPIV3. Cells are harvested after 24 hours and assayed for reporter activity.


T7
Promoter
...PUC19
Bicistronic Genome Analog DNA
PUC19...
BsaI
Transcribe
Bicistronic RNA
5'
Transfect
HPIV3-
infected cells
Harvest
CAT or luc assays

temperature. Each sample was placed immediately into the scintillation counter, and luminescence was measured over a one minute period. Lyophilized luciferase (Boehringer Mannheim) resuspended to 20 pg/μL was used as a positive control for the assay.

3. Protein Assay

Protein assays were performed according to the Micro-assay procedure using the Bio-Rad Protein Assay dye reagent concentrate (Biorad). 1 μL cell lysate (III.H.1) was diluted to 800 μL with water. 200 μL of the protein assay dye reagent were added, and absorbance was measured at 560 nm. Values were compared against a standard curve prepared with known concentrations of bovine serum albumin (BSA).

I. RNA Analysis

1. Isolation

RNA was prepared from wells of 6-well plates. Cells were transferred to microfuge tubes, pelleted and washed with cold TBS. The RNA extraction procedure followed the guanidinium thiocyanate-acid phenol method of Chomczynski and Sacchi (1987). Alternatively, TRIzol™ Reagent (Gibco/ BRL) was used. RNA was resuspended in a final volume of 20 μL DEPC-H_20 per well. 2 U RNAse-free DNAse 1 were added and samples were incubated at 37°C for 30 minutes. RNA quality was assessed by agarose gel electrophoresis.

2. Riboprobe Preparation

A 494 bp fragment (*Bsp*D1/*Pvu*II) was isolated from pPIV-LC-NP/P and cloned into pGem7 that had been digested with *Sma*1 and *Bsp*D1. This fragment contains approximately 350 nt of the luc gene, 130 nt of the CAT gene and spans the gene junction. Transcription *in vitro* yielded a negative sense probe. As well, a linearized DNA, pTRICAT (Ambion), was purchased that would allow synthesis of a negative sense CAT probe representing a different region of the CAT orf.

A 20 μL *in vitro* transcription reaction typically contained 5 μM [α-^{32}P] CTP (800 Ci/mmol, Amersham), though transcription of longer sequences was performed with an additional 5 μM unlabelled CTP. Reactions were incubated one hour at 37°C. 2 units of RQ1 DNAse (Promega) were added, and incubation was continued for 20 minutes at 37°C. 20 μL formamide gel loading buffer (80% formamide, 2mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) stopped the reactions, and RNA was denatured for 5 minutes at 95°C. Samples were loaded on a 8M urea, 5% polyacrylamide gel which was run for 2 hours at 200 volts in TBE buffer. The wet gel was exposed briefly to film, and the radioactive band was eluted overnight in 300 μL elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.2 % SDS; Ambion).

3. Northern Analysis

RNA was resolved on a formaldehyde gel, prepared according to Sambrook *et al.* (1989). Each sample contained approximately 20 μg of total cellular RNA. 50 ng of pPIV3-CAT(+) *in vitro* transcribed RNA was used as a positive control for hybridization with the negative sense RNA probe. Electrophoresis was carried out until the bromophenol blue was three-quarters of the way down the gel. The gel was soaked three times for thirty minutes in DEPC-H_2O. Lanes containing RNA markers (Gibco/BRL) were removed and stained in ethidium bromide. The rest of the gel was soaked in 0.05 N NaOH for twenty minutes, then in 20x SSC for 45 minutes.

Transfer of the RNA to nylon membrane was accomplished by capillary transfer in 20x SSC (Sambrook *et al.*, 1989). Zeta-Probe (Biorad) membrane was soaked for 10 minutes in 20x SSC before overnight transfer.

Hybridization was performed according to Zeta-Probe blotting membrane protocols (Biorad). The membrane was rinsed in 6x SSC to remove any traces of agarose. A five minute wash in 0.05 N NaOH replaced the baking or ultraviolet crosslinking step (Noonberg *et al.*, 1994). The membrane was then prewashed for 40 minutes in 0.1x SSC, 0.1% SDS. Prehybridization of the membrane was done at 42°C for twelve hours in 5x SSC, 50 mM sodium phosphate pH 6.5, 10x Denhardts', 1 mg/mL sonicated, denatured salmon sperm DNA, 50% formamide. The prehybridization and hybridization steps

were performed in a hybridization oven (Bellco). Hybridization was done in a similar solution containing 2x Denhardts', 100 μg/mL salmon sperm DNA, 25 mM sodium phosphate and $1x10^6$ cpm/mL ^{32}P-labelled RNA. Hybridization was allowed to proceed for 24 hours at 42°C. After hybridization, the blot was washed one hour in 2x SSC, 0.1% SDS at room temperature, 30 minutes in 0.1x SSC, 0.1% SDS at room temperature followed by a final wash in 0.1x SSC, 0.1% SDS at 55°C for 30 minutes. The membrane was wrapped in saran wrap before autoradiography.

Hybridization with ^{32}P-end labelled oligonucleotides was also attempted. Oligos 3061 and 3062, as negative and positive sense probes respectively (See Appendix), were labelled at their 5' termini with 5 U T4 polynucleotide kinase and 50 pmol [γ-^{32}P] ATP (3000 Ci/mmol, Amersham) for thirty minutes. Unincorporated label was removed by three rounds of precipitation with 2M ammonium acetate and ethanol. Prehybridization was performed in 6x SSC, 5x Denhardt's, 100 μM denatured salmon sperm DNA at 65°C for 4 hours. Hybridization was carried out in the same buffer at 40°C for two hours. Four 5 minute washes in 6x SSC, 0.1% SDS were performed at room temperature, and background levels of the blots were monitored with a Geiger counter. If necessary, a final wash in 2x SSC, 0.1% SDS was performed at the hybridization temperature.

4. Ribonuclease Protection

Ribonuclease protection assays have been reported to be more sensitive than Northern or even slot blot analyses (Kenney and Oglesbee, 1994), and can easily be made quantitative (Durnam and Palmiter, 1983; Hatada *et al.*, 1989). The RNase protection assays were performed with the aid of an RPA II Ribonuclease Protection Assay Kit (Ambion). The probe used, pTRICAT, was prepared as for Northern blots. $1x10^5$ cpm probe was annealed overnight at 45°C in hybridization buffer (80% formamide, 100 mM sodium citrate pH 6.4, 300 mM sodium acetate pH 6.4, 1 mM EDTA; Ambion) with total cellular RNA from two wells of a 6 well plate of infected and transfected 293 cells (approximately $2x10^6$ cells = 20 μg RNA). Single stranded RNA was digested with 4 U RNAse A and 20 U RNAse T1 for 30 minutes at 37°C. After RNAse inactivation and isopropanol precipitation, protected fragments were resolved on 8M urea, 5% acrylamide gels. Gels were fixed in 10% methanol, 10% acetic acid for thirty minutes and dried for two hours. Dry gels were exposed to X-ray film for several days.

IV. RESULTS

A. Bicistronic Constructs

cDNAs encoding HPIV3 vRNA analogs in which the viral genes had been replaced by the CAT and luc reporter genes were constructed (pPIV-CL see Figure 4; pPIV-LC see Figure 5). These plasmids each contain a T7 promoter immediately adjacent to the HPIV3 trailer as well as a *Bsa*1 site directly abutting the HPIV3 leader. Transcription of *Bsa*1-digested plasmids was predicted to yield negative sense RNA consisting of (in the 3′ to 5′ direction) the 55 nt HPIV3 leader, 10 nt NP gene start, 46 nt non-translated NP sequence, CAT or luc coding sequence, 21 nt non-viral cloning region, luc or CAT coding sequence, 58 nt L gene non-translated sequence, 13 nt L gene end and the 44 nt HPIV3 trailer. The genome analogs transcribed from pPIV-CL and pPIV-LC are 2649 and 2652 nt, respectively. The difference is due to the remnants of an *Xba*1 site in pPIV-CL and the remnants of a *Pst*1 site in pPIV-LC. These transcribed regions represent approximately 17.2% of the HPIV3 genome (15 463 nt), and contain 226 nt of HPIV3 terminal sequences that have been shown to be both necessary and sufficient for transcription and replication (Dimock and Collins, 1993).

Two approaches were used to create mutant constructs. Direct cloning of complementary, annealed oligonucleotides

Figure 4 Construction of pPIV-CL from pPIV3-CAT(-). The positions of the HPIV3 leader (*l*) and trailer (*t*) are shown, as are the nucleoprotein (NP) gene start (gs) and non-coding (nc) sequences and the polymerase (L) non-coding (nc) and gene end (ge) sequences. The T7 promoter and the CAT orf are also indicated (black boxes). Synthesized oligonucleotides shown at the top of the figure were annealed and cloned into the *Pst*I site of pPIV3-CAT(-). Both this modified version of pPIV3-CAT(-) and pGEM-luc were digested with *Bam*HI and *Xho*I. Ligation yielded pPIV-CL, which retains the *Sac*II, *Hpa*I and *Bam*HI sites at the junction between the CAT and luc orfs. Transcription off the T7 promoter of *Bsa*I-digested pPIV-CL yields a negative sense RNA that is 2649 nt in length. The drawing is not to scale.

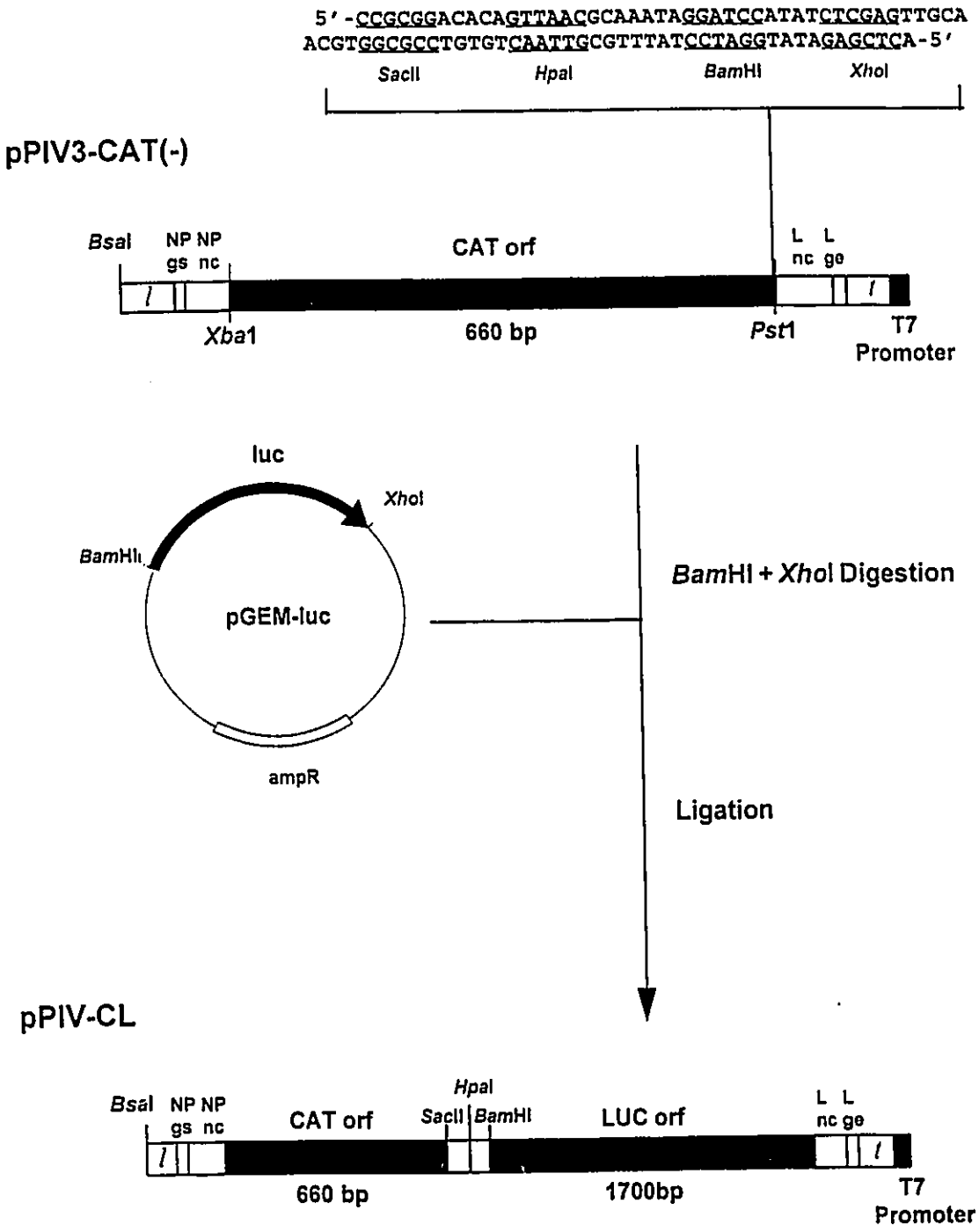
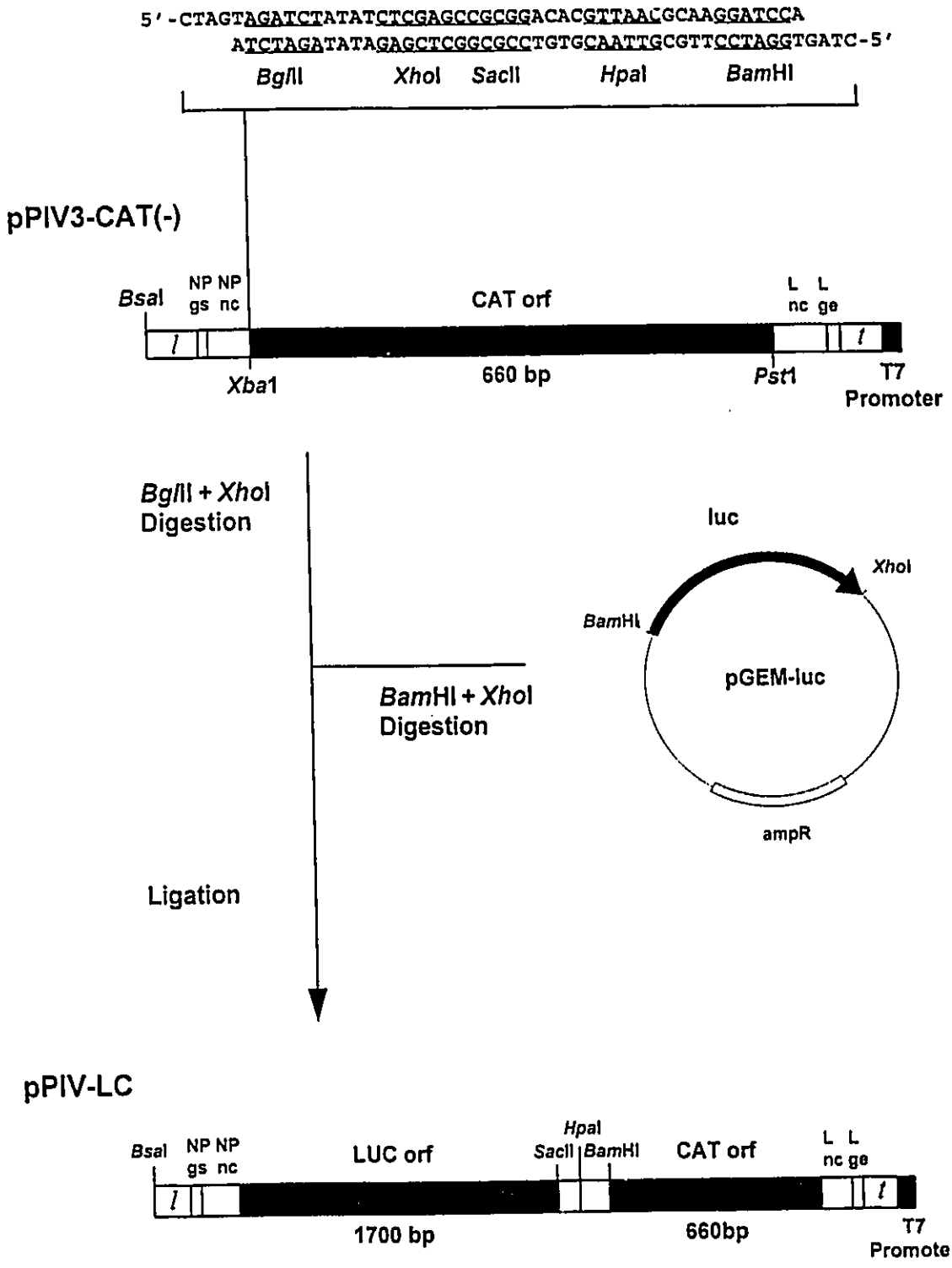

5'-CCGCGGACACAGTTAACGCAAATAGGATCCATATCTCGAGTTGCA
ACGTGGCGCCTGTGTCAATTGCGTTTATCCTAGGTATAGAGCTCA-5'
SacII
HpaI
BamHI
XhoI
pPIV3-CAT(-)
BsaI
NP NP
gs nc
CAT orf
L L
nc ge
Xba1
660 bp
Pst1
T7
Promoter
luc
XhoI
BamHI
pGEM-luc
ampR
BamHI + XhoI Digestion
Ligation
pPIV-CL
BsaI
NP NP
gs nc
CAT orf
HpaI
SacII
BamHI
LUC orf
L L
nc ge
660 bp
1700bp
T7
Promoter

Figure 5 Construction of pPIV-LC from pPIV3-CAT(-). The positions of the HPIV3 leader (*l*) and trailer (*t*) are shown, as are the nucleoprotein (NP) gene start (gs) and non-coding (nc) sequences and the polymerase (L) non-coding (nc) and gene end (ge) sequences. The T7 promoter and the CAT orf are also indicated (black boxes). Synthesized oligonucleotides shown at the top of the figure were annealed and cloned into the *Xba*I site of pPIV3-CAT(-). The modified pPIV3-CAT(-) was then digested with *Bgl*II and *Xho*I, and pGEM-luc was digested with *Bam*HI and *Xho*I. Ligation yielded pPIV-LC, which retains the *Sac*II, *Hpa*I and *Bam*HI sites at the junction between the luc and CAT orfs. Transcription off the T7 promoter of *Bsa*I-digested pPIV-LC yields a negative sense RNA that is 2652 nt in length. The drawing is not to scale.


5'-CTAGTAGATCTATATCTCGAGCCGCGGACACGTTAACGCAAGGATCCA
ATCTAGATATAGAGCTCGGCGCCTGTGCAATTGCGTTCCTAGGTGATC-5'
BglII
XhoI
SacII
HpaI
BamHI
pPIV3-CAT(-)
BsaI
NP NP
gs nc
CAT orf
L L
nc ge
XbaI
660 bp
PstI
T7
Promoter
BglII + XhoI
Digestion
luc
XhoI
BamHI
pGEM-luc
ampR
BamHI + XhoI
Digestion
Ligation
pPIV-LC
BsaI
NP NP
gs nc
LUC orf
HpaI
SacII
BamHI
CAT orf
L L
nc ge
1700 bp
660bp
T7
Promoter

between the *Bam*HI and *Sac*II sites of pPIV-CL or pPIV-LC yielded the initial set of mutants. Site-directed mutagenesis using the two constructs with the NP/P natural junction (pPIV-CL-NP/P and pPIV-LC-NP/P) created the remaining bicistronic constructs (Table 3). All clones were sequenced across the mutated region. An example of sequencing data from one construct is also provided (Figure 6).

B. Expression of Reporter Genes

To test whether the two constructs containing the HPIV3 NP/P gene junctions (pPIV-CL-NP/P; pPIV-LC-NP/P) expressed both reporter genes, 293 cells were infected with HPIV3 and transfected with each vRNA analog. After an overnight incubation, cells were harvested and assayed for CAT and luc activities. Basic requirements for rescue adhered to the observations made for pPIV3-CAT(-) (Dimock and Collins, 1993); i.e. rescue required helper virus infection and reporter activity depended on transfection with synthetic vRNA.

Luciferase assays were performed on all of the mutants to confirm CAT data. Luciferase is a more sensitive reporter than CAT, but did not yield results that were as reproducible as for the CAT gene. Thus, CAT became the reporter of choice, while luciferase was only employed to test certain

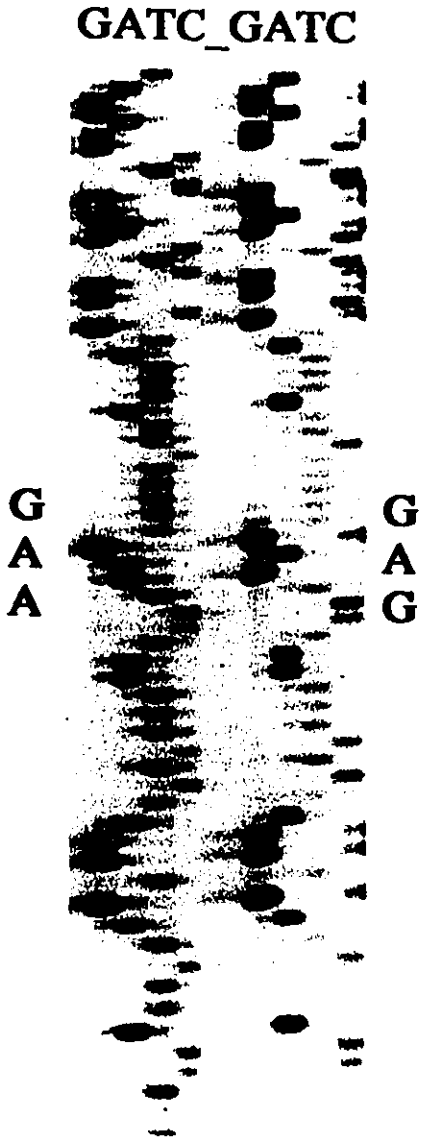
Table 2 Constructs used for rescue experiments.

Construct Name	Description	Rule of Six[2]
pPIV3-CAT(-)	monocistronic CAT (-ve sense)	no
pPIV3-CAT(+)	monocistronic CAT (+ve sense)	no
pPIV-Luc	monocistronic Luc	no
pPIV-ßGal	monocistronic ßGal	no
pPIV-CL[1]	no viral sequences in junction	no/yes[3]
pPIV-CL-NP/P[1]	NP/P (P/M) natural junction	no
pPIC-CL-F/HN[1]	F/HN natural junction	no
pPIV-CL-HN/L[1]	HN/L natural junction	no
pPIV-CL-M/F[1]	M/F natural junction	yes/no[3]
pPIV-CL-M/FΔ8[1]	M/F junction with 8 nt deleted	no
pPIV-CLΔGE[1]	NP/P junction; gene end deleted	no
pPIV-CLΔGAA[1]	NP/P junction; Ig deleted	no
pPIV-CLΔGS[1]	NP/P junction; gene start deleted	no/yes[3]
pPIV-CL+GAA[1]	NP/P junction; extra Ig inserted	no
pPIV-CLΔ53-55[1]	CTT from leader deleted	no
pPIV-CL-IgGUA[1]	NP/P junction; Ig changed to GUA	no
pPIV-CL-IgGGA[1]	NP/P junction; Ig changed to GGA	no
pPIV-CL-IgAAA[1]	NP/P junction; Ig changed to AAA	no
pPIV-CL-IgGAG[1]	NP/P junction; Ig changed to GAG	no

pPIV-CL-IgGA[1]	NP/P junction; Ig changed to GA	no
pPIV-CL-IgAA[1]	NP/P junction; Ig changed to AA	no
pPIV-CL-IgA[1]	NP/P junction; Ig changed to A	no
pPIV-CL-IgG[1]	NP/P junction; Ig changed to G	no
pPIV-CL-GE+U[1]	NP/P junction; 1 U added to GE	no/yes[3]
pPIV-CL-GEΔU[1]	NP/P junction; 1 U deleted from GE	no
pPIV-CL-GEΔ2U[1]	NP/P junction; 2 U's deleted	no
pPIV-CL-GEΔ3U[1]	NP/P junction; 3 U's deleted	no
pPIV-CL-GEΔ4U[1]	NP/P junction; 4 U's deleted	yes/no[3]
pPIV-CL-NP/PΔ1871-2523	652 bp deletion in luc	yes
pPIV-CL-NP/P+3	3 bp insertion in luc	no
pPIV-CL-NP/P+2	2 bp insertion in luc	yes
pPIV-CL-GEΔ4U+3	3 bp insertion in luc of Δ4U mutant	no
pPIV-CL-GE+U+3	3 bp insertion in luc of +U mutant	no

[1] Identical mutations were introduced in pPIV-LC constructs.
[2] Indicates whether or not the length of the transcribed minigenome is a multiple of 6 nt.
[3] The LC construct is listed second.

Figure 6 Sequencing to confirm mutations. On the left is the sequence of control DNA, pPIV-CL-NP/P, and the sequence on the right is the mutant pPIV-CL-IgGAG. Sequence lanes are shown in the order GATC. The sequence of the intergenic regions are depicted.


GATC_GATC
G
A
A
G
A
G

'Rule of Six' constructs (see below). However, this bicistronic system should still be capable of providing a choice of reporter genes for future analyses.

1. Natural gene junctions function equally well.

Constructs were made that contained each of the five naturally occurring HPIV3 gene junctions. The sequences of these junctions are shown in Table 1. CAT activity was measured for constructs in which the CAT orf was located in either the upstream (Fig. 7a) or the downstream (Fig. 7b) position relative to the junction. While Figure 7 depicts a representative picture of the CAT assay results, data from several experiments were obtained by counting radioactive spots from TLC plates in a scintillation counter and correcting for total protein in each sample. Table 4 contains the CAT activity for all minigenomes expressed relative to the corresponding NP/P genome analogs, which were arbitrarily assigned the value 100.

All of the natural junctions appeared to direct expression of the CAT gene equally well - with the exception of one. The anomaly observed in this initial set of experiments was for the minigenomes containing the M/F junction (pPIV-CL-M/F; pPIV-LC-M/F). This junction is different from the others in that the M gene end sequence contains 8 extra nucleotides. When the CAT orf was situated

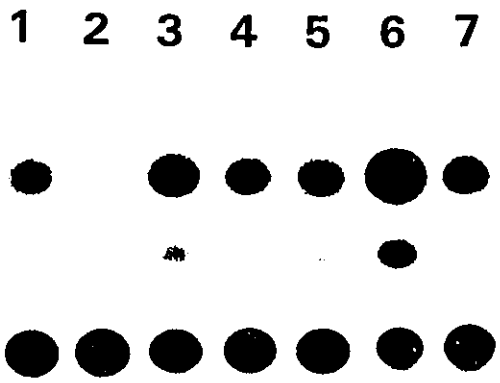
Table 3. CAT assay results expressed as activity relative to the NP/P junction. (n=3)

Junction	Relative CAT Activity	
	Upstream	Downstream
no viral sequences	5±1[1]	4±3[2]
NP/P	100	100
F/HN	108±43	90±8
HN/L	100±32	133±34
M/F	1200±580[2]	52±30
M/FΔ8	238±50	18±10
GE deleted	17±3	7±7
GS deleted	1275±90	1±0.4[2]
Ig deleted	200±50	4±4
extra Ig inserted	1006±96	4±3
GAA from leader deleted	3±1	2±1
Ig changed to GUA	33±15	133±5
Ig changed to GGA	25±10	193±82
Ig changed to AAA	15±4	112±13
Ig changed to GAG	15±2	123±72
Ig changed to GA	5±0.5	10
Ig changed to AA	16±0.5	6±5
Ig changed to G	35±12	5±2
Ig changed to A	35±32	10±4
+1U in GE	200±32	313±95[2]
-1U in GE	5±2	20±7
-2Us in GE	6±2	10±2
-3Us in GE	12±4	11±2
-4Us in GE	60±16[2]	9±1

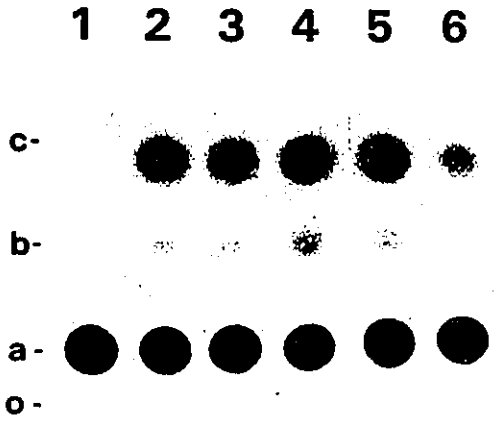
[1] standard deviation.
[2] minigenome which has a length that is a multiple of six.

Figure 7 **CAT expression with natural junctions.** CAT activity in 293 cells transfected with bicistronic minigenomes containing a CAT orf (A) upstream of the natural HPIV3 junctions: (1) pPIV3-CAT (-), (2) pPIV-CL, (3) pPIV-CL-NP/P, (4) pPIV-CL-F/HN, (5) pPIV-CL-HN/L, (6) pPIV-CL-M/F, and (7) pPIV-CL-M/FΔ8, and (B) downstream of the natural HPIV3 junctions: (1) pPIV-LC, (2) pPIV-LC-NP/P, (3) pPIV-LC-F/HN, (4) pPIV-LC-HN/L, (5) pPIV-LC-M/F and (6) pPIV-LC-M/FΔ8. O indicates the origin, a indicates the unacetylated chloramphenicol and b and c refer to the different monoacetylated forms of chloramphenicol.

A



B



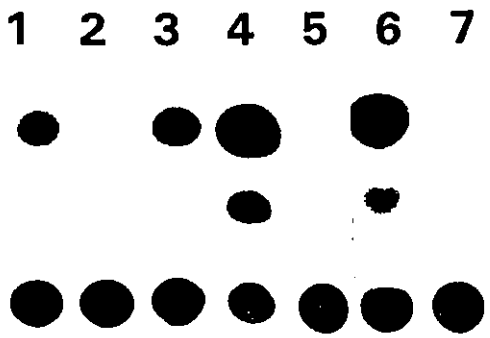
upstream of the M/F junction, CAT expression was increased approximately 10 fold over that of the minigenome containing the NP/P junction. The genome analog transcribed from pPIV-CL-M/F has a length that is divisible by six, so the 'Rule of Six' may explain this data (see Section IV.B.6). When the 8 extra nucleotides were removed (pPIV-CL-M/FΔ8), activity returned to close to control levels. When the CAT gene was placed in the downstream position, normal activity was observed. If the 8 nucleotides were removed (pPIV-LC-M/FΔ8), a 5 fold decrease in CAT expression was observed.

2. Deletion of junction elements has variable effects.

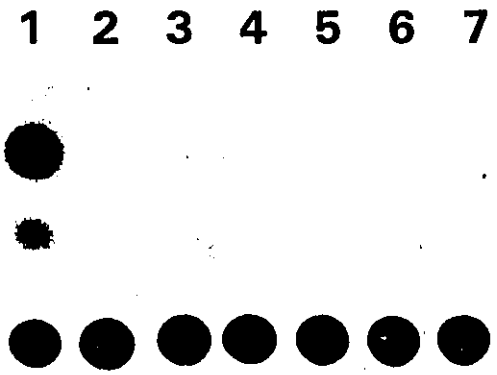
Initial experiments to elucidate the roles of the separate junctional elements used constructs that had deletions or duplications of specific junctional sequences. Deletion of the gene end (pPIV-CLΔGE) from the junction decreased expression of the CAT gene, located in either the upstream or downstream position, to background levels. In order to maintain the relative positioning of the gene end and intergenic sequences, as well as the intergenic and gene start sequences, but to change the spacing between the gene end and gene start sequences, a second intergenic trinucleotide was inserted. RNA transcribed from pPIV-CL+GAA resulted in 10 fold higher expression of the CAT gene when it was in the upstream position but no detectable expression of

Figure 8 Effects of gross deletions of junctional elements on CAT activity. CAT activity in 293 cells transfected with minigenomes from the constructs containing the CAT gene in (A) the upstream position: (1) pPIV-CL-NP/P, (2) pPIV-CL, (3) pPIV-CLΔGAA, (4) pPIV-CLΔGS, (5) pPIV-CLΔGE, (6) pPIV-CL+GAA , (7) pPIV-CLΔ53-55, and (B) the downstream position: (1) pPIV-LC-NP/P, (2) pPIV-LC, (3) pPIV-LCΔGAA, (4) pPIV-LCΔGS, (5) pPIV-LCΔGE, (6) pPIV-LC+GAA and (7) pPIV-LCΔ53-55.

A



B

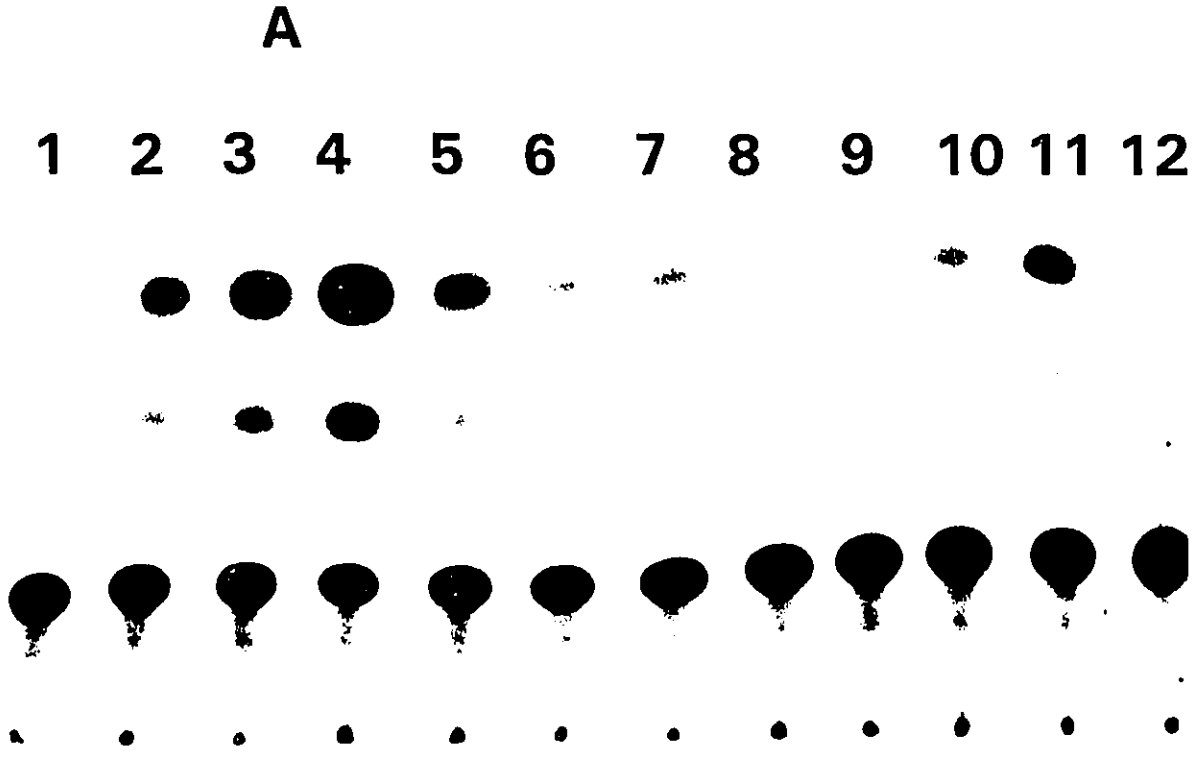


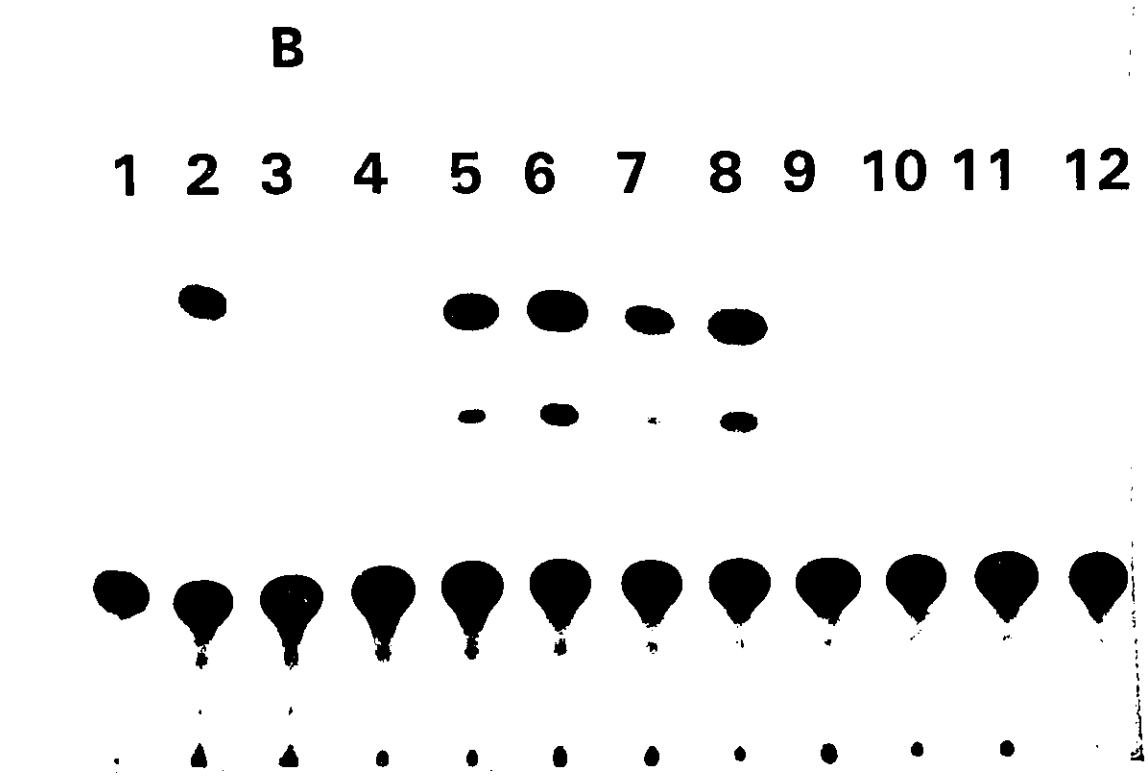
CAT when it was the downstream gene. Similarily, deletion of the gene start sequence at the junction of the CAT and luc cistrons (pPIV-CLΔGS) was seen to increase expression of the CAT gene from the upstream position more than 10 fold but to abolish CAT expression from the downstream position (Figure 8). Removal of the intergenic trinucleotide (pPIV-CLΔGAA) had little effect on the expression of the CAT gene from the upstream position, but if CAT was in the downstream position, its expression was abrogated. Genome analogs which had the intergenic equivalent in the leader sequence removed (pPIV-CLΔ53-55; pPIV-LCΔ53-55) did not lead to expression of CAT activity when CAT was placed either upstream or downstream of the NP/P junction (Figure 8).

3. Mutational analysis of the intergenic trinucleotide.

A role for the nontranscribed trinucleotide has not yet been determined. However, its importance in transcription is suggested by its high degree of conservation and was evident when it was deleted, as expression of the CAT gene from the downstream position decreased (Figure 9). To more closely examine this sequence, a set of constructs was made that had alterations in the length and the sequence of the intergenic trinucleotide. When the CAT gene was in the upstream position, CAT expression was reduced for all mutants (Figure 9A), though most mutations were tolerated to some degree.

Figure 9 Examination of the role of the intergenic trinucleotide in transcription. CAT expression in 293 cells transfected with minigenomes from the constructs containing the CAT gene in (A) the upstream position: (1) pPIV-CL, (2) pPIV-LC-NP/P, (3) pPIV-CLΔGAA, (4) pPIV-CL+GAA, (5) pPIV-CL-IgGUA, (6) pPIV-CL-IgGGA, (7) pPIV-CL-IgGGG, (8) pPIV-CL-IgGAG, (9) pPIV-CL-IgGA, (10) pPIV-CL-IgAA, (11) pPIV-CL-IgA, (12) pPIV-CL-IgG, and (B) the downstream position: (1) pPIV-LC, (2) pPIV-LC-NP/P, (3) pPIV-LCΔGAA, (4) pPIV-LC+GAA, (5) pPIV-LC-IgGUA, (6) pPIV-LC-IgGGA, (7) pPIV-LC-IgAAA, (8) pPIV-LC-IgGAG, (9) pPIV-LC-IgGA, (10) pPIV-LC-IgAA, (11) pPIV-LC-IgA and (12) pPIV-LC-IgG.


A
1 2 3 4 5 6 7 8 9 10 11 12

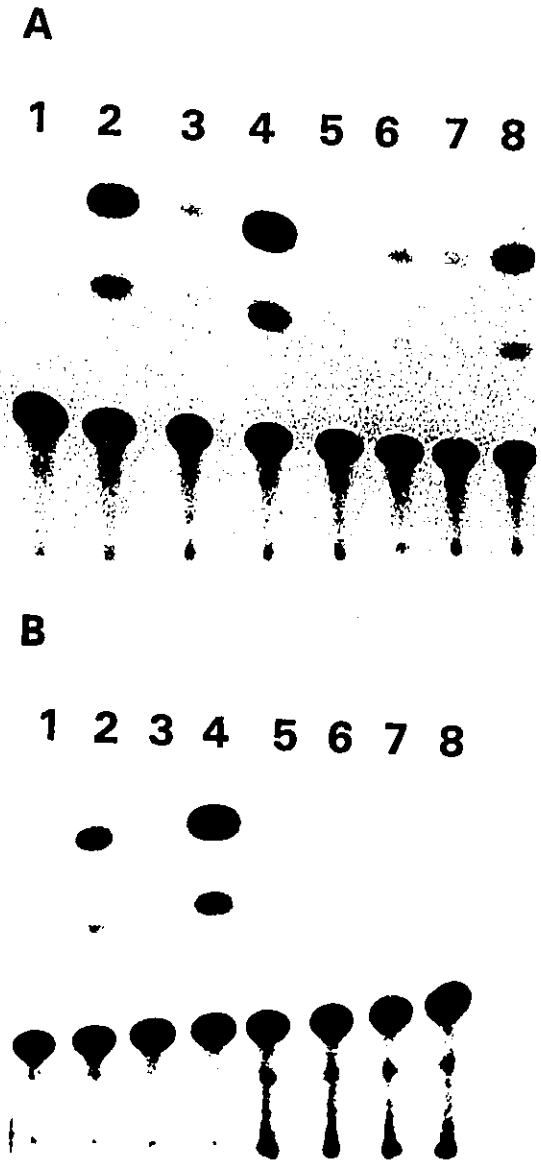

B
1 2 3 4 5 6 7 8 9 10 11 12

When the CAT gene was in the downstream position, CAT expression appeared to be affected more by spacing than by the actual sequence (Figure 9B). Changing the intergenic trinucleotide to GUA, GGA, AAA or GAG had no effect on CAT activity. However, any mutation changing the length of the trinucleotide abolished expression of the CAT gene when it was located in the downstream position.

4. The poly U tract of the gene end sequence cannot be shortened.

Specific mutations were made to the poly U stretch in the gene end sequence (the sequence presumed to be the template for polyadenylation), resulting in U tracts between one and six nucleotides long. CAT activity was slightly increased (2-3 fold) with the addition of an extra U to this presumed polyadenylation signal for minigenomes when the CAT gene was in either the upstream or the downstream position (pPIV-CL-GE+U; pPIV-LC-GE+U). However, shortening the U stretch even by one nucleotide (pPIV-CL-GEΔU; pPIV-LC-GEΔU) had detrimental effects on CAT gene expression (Figure 10). One mutant minigenome, PIV-CL-GEΔ4U, showed close to normal levels of CAT expression; however, the length of this minigenome is a multiple of six nt (see Section IV.B.6).

Figure 10 An examination of the putative polyadenylation signal of the gene end sequence. CAT activity in 293 cells transcribed with minigenomes from the constructs containing the CAT gene in (A) the upstream position: (1) pPIV-CL, (2) pPIV-CL-NP/P, (3) pPIV-CLΔGE, (4) pPIV-CL+U, (5) pPIV-CLΔU, (6) pPIV-CLΔ2U, (7) pPIV-CLΔ3U, (8) pPIV-CLΔ4U, and (B) the downstream position: (1) pPIV-LC, (2) pPIV-LC-NP/P, (3) pPIV-LCΔGE, (4) pPIV-LC+U, (5) pPIV-LCΔU, (6) pPIV-LCΔ2U, (7) pPIV-LCΔ3U, (8) pPIV-LCΔ4U.


A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8

5. Passage confirms packaging.

Passing of the culture supernatants was performed to confirm that genome analogs were packaged into transmissible particles. 293 cells were infected with clarified culture supernatant from transfected, HPIV3-infected cells for one hour and then superinfected for one hour with helper HPIV3. The same patterns of CAT and luc activities were observed after passage; i.e. positive cultures remained positive and negative cultures remained negative (data not shown).

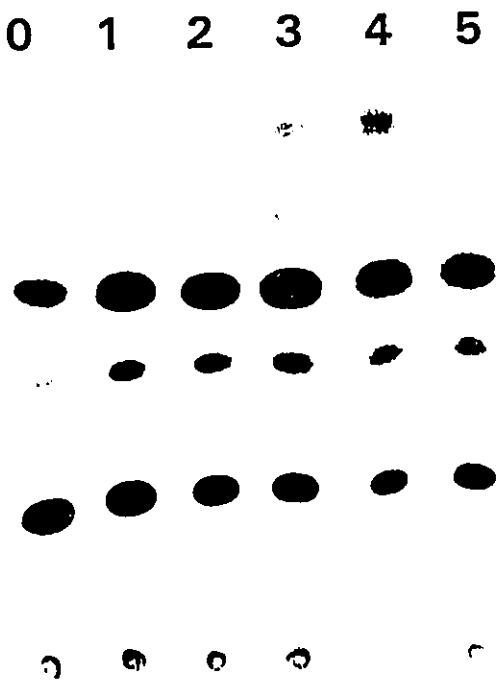
Serial passage of the culture supernatants was also performed, with wild-type helper virus superinfection at each passage. Upon serial passage of the culture supernatants, a variety of outcomes were observed (Figure 11). Most commonly, serial passage resulted in signal amplification (Figure 11A), confirming earlier data for pPIV3-CAT(-) derived minigenomes (Dimock and Collins, 1993). However, the signal was occasionally seen to decline until all activity was lost (Figure 11B). Most interesting was the observation that gene expression could increase significantly during one passage (Figure 11C).

6. The Rule of Six has a role in rescue.

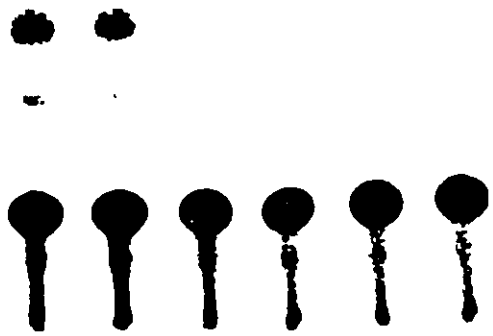
It was noted that several minigenomes led to unexpectedly high levels of CAT expression upon transfection

Figure 11 Serial passage of packaged HPIV3 RNA analogs. RNA transcribed from pPIV-CL-NP/P was used to transfect 293 cells. After 24 h, culture supernatant was removed, clarified and used to infect fresh cells. Cells were harvested after each pass. Upon passage, CAT activity is typically seen to increase (A), although activity was also seen to decrease (B), or increase substantially during one pass (C). In each panel, 0 refers to the inital transfection, and 1-5 refer to the pass number. The diacetylated form of chloramphenicol is indicated (x).

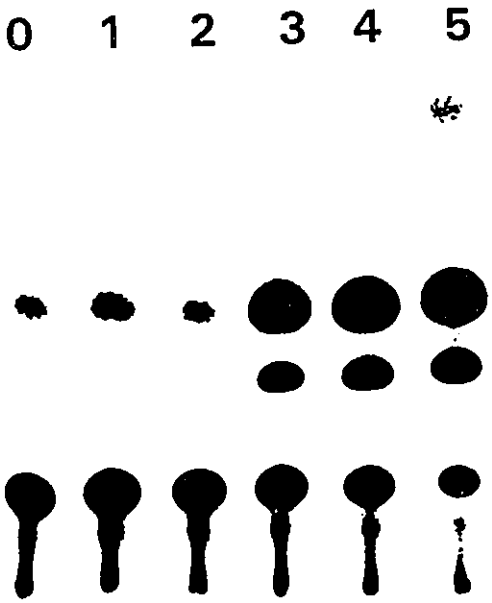
A.



B.



C.

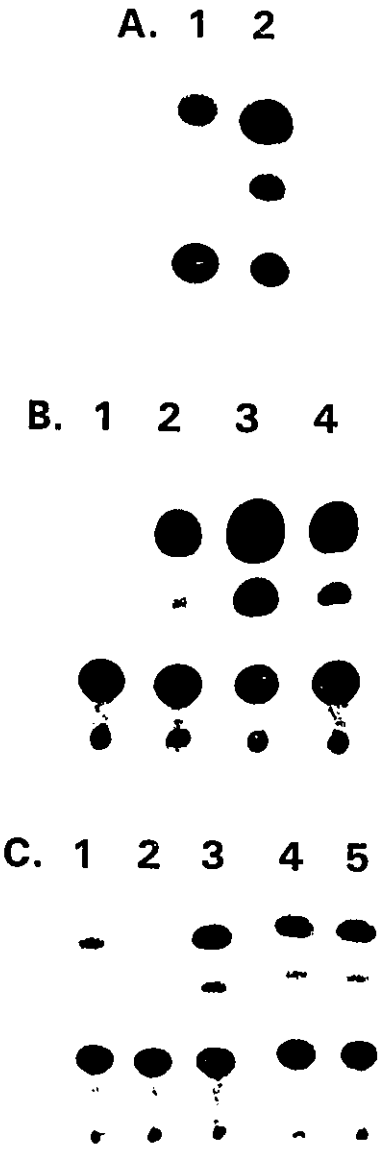


of 293 cells (Table 4). All of these minigenomes had lengths that were divisible by 6. These observations suggested that minigenomes that were multiples of 6 nt were rescued more efficiently that those that were not.

To test the `Rule of Six' without altering potential *cis*-acting sequences, the luc coding region of pPIV-CL-NP/P was selected as a site for mutations that would alter the overall length of the genome analogs. pPIV-CL-NP/PΔ1871-2523 contains a 652 bp deletion in the luc coding region which results in a minigenome that is evenly divisible by 6. CAT expression for this minigenome is increased 5-10 fold (Figure 12A). pPIV-CL-NP/P+3 and pPIV-CL-NP/P+2 were constructed by linearizing within the luc sequences at the *Bsu*361 and *Bsp*DI sites, respectively, filling in the sticky ends and religating. The pPIV-CL-NP/P+2 RNA is a multiple of 6 while that for pPIV-CL-NP/P+3 is not. The minigenome from pPIV-CL-NP/P+3 results in close to normal levels of CAT expression while the minigenome from pPIV-CL-NP/P+2 results in a 5-10 fold increase in CAT expression (Figure 12B).

Several minigenomes constructed to address other questions were also divisible by 6 (Table 2). One of these constructs (pPIV-CL-GEΔ4U) was mutated within the luc coding region to alter the length of the RNA. Figure 12C shows that changing the length of the pPIV-CL-GEΔ4U minigenome by three nucleotides reduces CAT activity to expected levels (in this

Figure 12 Evidence for the 'Rule of Six'. Panel A shows CAT expression for the control pPIV-CL-NP/P (1) and the mutant pPIV-CLΔ1871-2523 (2). Panel B shows CAT expression for the negative control pPIV-CL (1), pPIV-CAT-NP/P (2), pPIV-CL+2 (3) and pPIV-CL+3. In panel C, mutations similar to that in pPIV-CL+3 were made to two of the other genome anologs. The minigenome transcribed from pPIV-CL-GEΔ4U is divisible by six (1); the one from pPIV-CL-GEΔ4U+3 is not (2). pPIV-CL-NP/P was run in lane 3. When three nucleotides were added at the same site as for pPIV-CL+3 to an analog that transcribed a minigenome whose length is not divisible by six (pPIV-CL-GE+U; lane (4), CAT expression did not change (pPIV-CL-GE+U+3; lane (5).


A. 1 2
B. 1 2 3 4
C. 1 2 3 4 5

case, to background). Making a similar mutation to pPIV-CL-GE+U, a construct that leads to synthesis of a minigenome that is not a multiple of 6 nt, yields an altered minigenome that is also not a mutiple of 6 nt, and has no effect on CAT expression (Figure 12c).

C. RNA Analysis

The results obtained by assaying CAT gene expression could be interpreted in several ways. For example, in cases where CAT activity was decreased, it was not evident if this was the result of polymerase readthrough, improper polyadenylation and termination, or failure to initiate. To distinguish between these possibilities, RNA from infected and transfected cells was analysed by Northern blot and ribonuclease protection.

1. RNA species detected in transfected cells by Northern blotting.

RNA from transfected cells was analysed by Northern blot to allow a visualization of the specific RNA species present. HPIV3-infected 293 cells were transfected with the RNA transcribed from various mutant constructs, and 20-24 hrs post-infection, RNA was isolated. Antigenome RNA and CAT mRNA species can be detected by Northern blotting using a

Figure 13 Northern blot analysis of RNA isolated from 293 cells transfected with different minigenome analogs. RNA was isolated, electrophoresed in formaldehyde agarose gels and hybridized as described in Section III.I.3. The negative sense probe contained luc and CAT sequences. (1) pPIV-CL-M/F RNA (2) pPIV-LC-NP/P RNA, (3) pPIV-luc RNA, (4) pPIV3-CAT(-) RNA, and (5) 10 ng *in vitro* transcribed pPIV3-CAT(+)(b). Nonspecific hybridization to 28S ribosomal RNA is indicated (a). CAT message is detected in all RNA samples except from pPIV-luc.

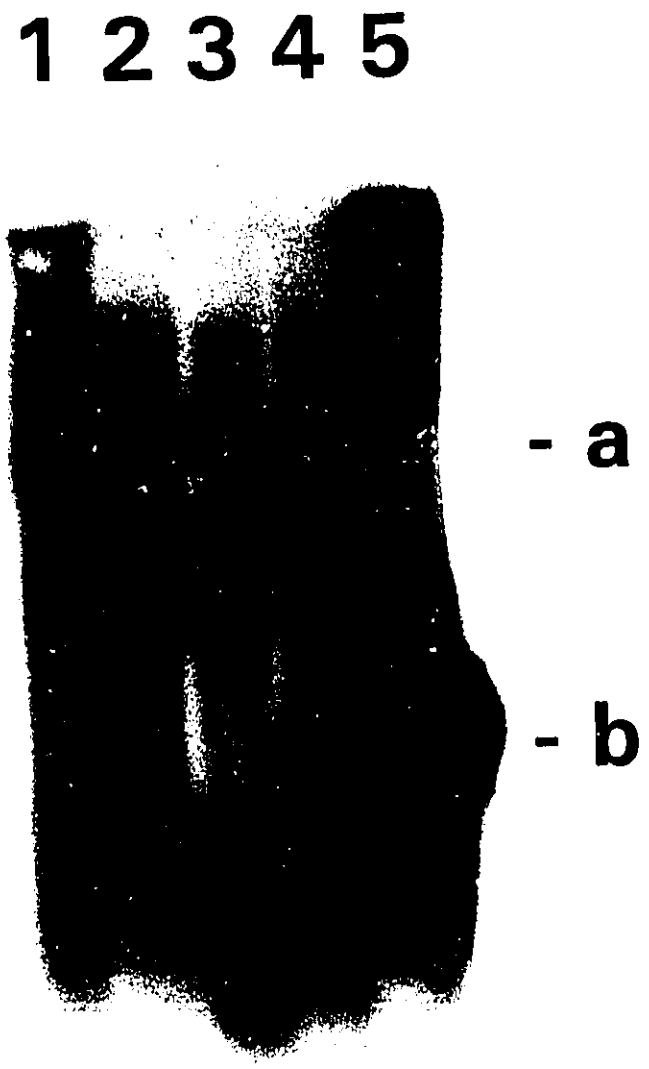
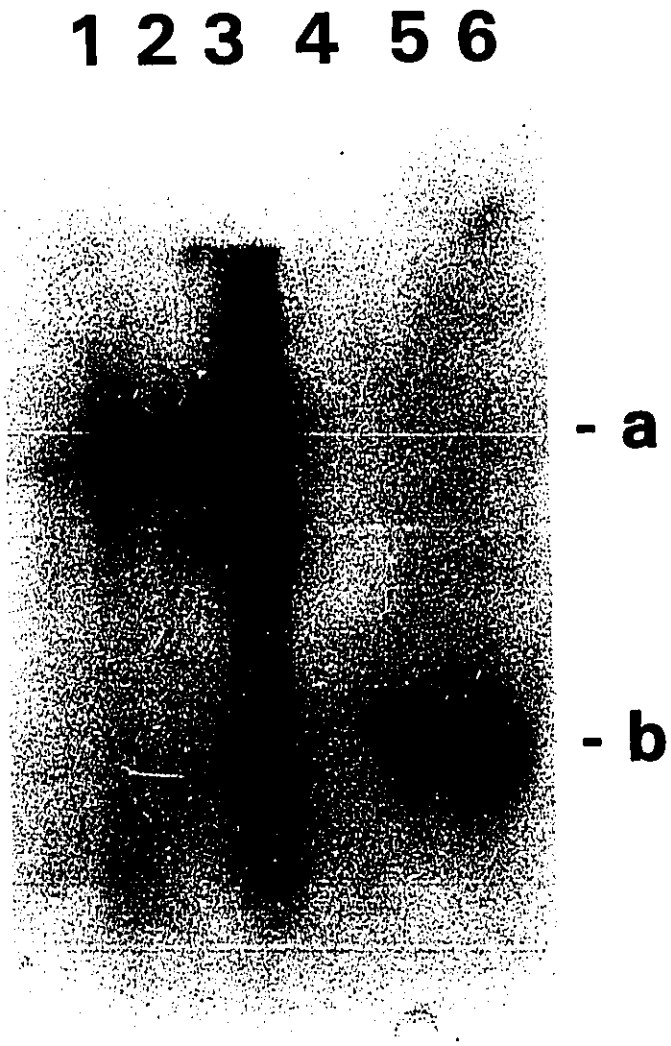

1 2 3 4 5
- a
- b

Figure 14 Northern blot analysis of RNA isolated from pPIV-CL-NP/P transfected, HPIV3-infected 293 cells. RNA was analysed as described in the legend to Figure 13. The negative sense probe was transcribed from pTRICAT. (1) 5 μg total cellular RNA, (2) 10 μg total cellular RNA, (3) 20 μg total cellular RNA, (4) no RNA, (5) 10 ng *in vitro* transcribed pPIV3-CAT(+) and (6) 100 ng *in vitro* transcribed pPIV3-CAT(+). Possible CAT message (b) and antigenome (a) species are marked.

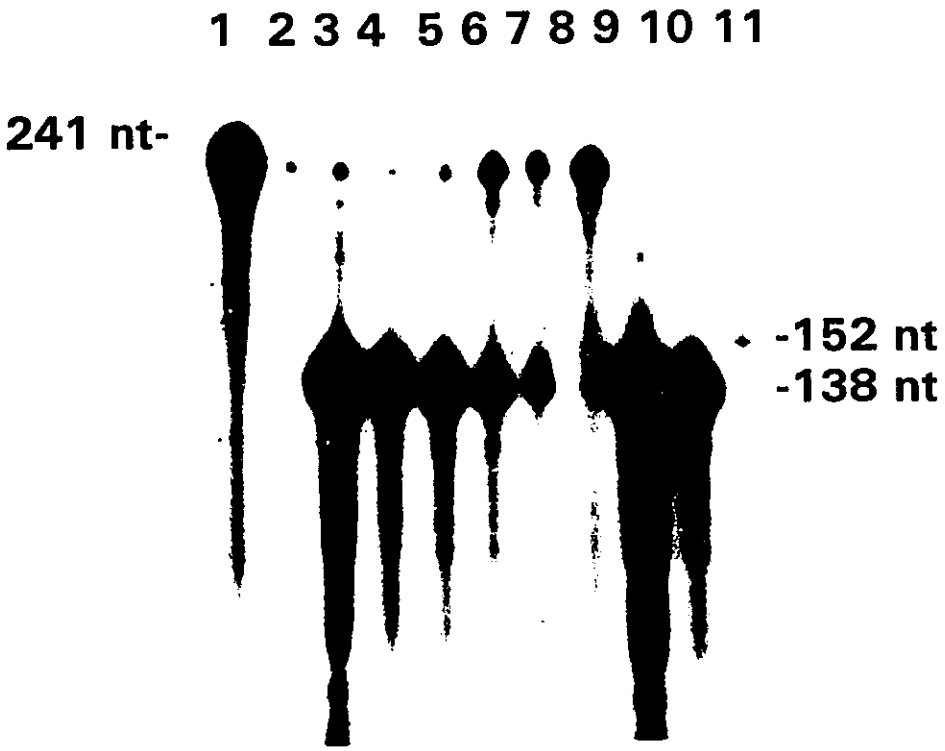

1 2 3 4 5 6
- a
- b

negative sense CAT probe (Figures 13 and 14). Antigenome RNA is ~2600 nt, and it runs between the 28S and 18S ribosomal RNA bands. The *in vitro* transcribed positive control CAT sequence is 660 nt. The wide band believed to be CAT RNA polyadenylated to various degrees appears specific as this band is absent from RNA isolated from cells transfected with the pPIV-luc minigenome, even after long exposure (Figure 13, lane 3). Northern blots performed with the luc-CAT riboprobe also displayed hybridization to cellular 28S ribosomal RNA (Figure 13,a). Nonspecific hybridization was partially alleviated by RNAse treatment of the blots and by use of an alternate, pTRICAT, probe (Figure 14).

2. Detection of CAT message by RNAse protection.

RNAse protection is a more sensitive technique than Northern blotting that allows quantitation of the individual species present. RNAse protection using a 241 nt, supplied, negative sense template for probe could easily detect as little as 5 pg of a 138 nt supplied, control, positive sense target RNA (Figure 15). A CAT specific band (152 nt) could also be detected from RNA from HPIV3-infected and pPIV-CL-NP/P minigenome-transfected cells (Figure 15, lane 11). The amount of specific RNA was too low for comparison between analogs or quantitation; however this was likely the result of low transfection efficiencies, not the detection method.

Figure 15 RNAse protection using control template and target RNA. (1) undigested probe (241 nt), (2) yeast RNA (-) control, (3) 400 pg, (4) 200 pg, (5) 100 pg, (6) 50 pg, (7) 20 pg, and (8) 5 pg (+) sense control CAT target RNA. (9-11) 20 μg total cellular RNA from cells transfected with pPIV-CL-NP/P. Hybridizations were spiked with (9) 400 pg (+) sense control CAT target RNA, (10) 200 pg (+) sense control CAT target RNA, or (11) no positive sense competitor. The sizes of each RNA species is given.


1 2 3 4 5 6 7 8 9 10 11
241 nt-
-152 nt
-138 nt

V. Discussion

The simplest model for paramyxovirus transcription predicts a 3′ terminal polymerase entry site and a polar gradient of transcription. The polymerase initially acts as a transcriptase, terminating and reinitiating at the end of the leader sequence and at each gene junction. Transcription is terminated and reinitiated by signals at the junctions between genes. The switch from transcription to replication is controlled at the level of chain initiation by the concurrent assembly of nascent RNA with NP to form RNP. This switch is part of a self-regulatory system, in which the concentration of unassembled NP determines the frequency of genome replication (Vidal and Kolakofsky, 1989). Primarily because of the inability to detect leader species in infected cells, a modified model for paramyxovirus transcription has been proposed (Blumberg *et al.*, 1991) in which replication and transcription start at a terminal and an internal auxiliary promoter site, respectively. This model suggests that transcription initiates at the beginning of the NP gene, after the leader sequence.

Until the recent development of rescue or 'reverse genetic' techniques, negative sense RNA viruses have been refractory to the introduction and recovery of engineered changes to genomic sequences. The main objective of this study was to use a rescue system for HPIV3 genome analogs for

the analysis of the roles of HPIV3 junctional elements in transcription. The first specific objective of this project was to synthesize bicistronic genome analogs containing the CAT and luciferase reporter genes. An array of cDNAs that would serve as templates for the transcription *in vitro* of synthetic analogs of HPIV3 were synthesized. Initially, two cDNAs containing the CAT and luc reporter genes flanked by HPIV3 terminal sequences (pPIV-CL; pPIV-LC) were constructed (see Figures 4 and 5). Subsequently, oligonucleotide pairs representing natural and altered HPIV3 gene junctions were cloned between the reporter genes. Constructs with NP/P junctions (pPIV-CL-NP/P; pPIV-LC-NP/P) were then chosen for further manipulation via site-directed mutagenesis.

The various HPIV3 genome analogs synthesized *in vitro* were transfected into HPIV3-infected 293 cells to examine the roles of the gene end, intergenic and gene start sequences in transcription. Transfection of HPIV3-infected cells with negative sense RNA, transcribed *in vitro* from the linearized bicistronic DNA constructs pPIV-CL-NP/P and pPIV-LC-NP/P, resulted in rescue. Expression of CAT and luc enzymatic activities indicated that transcription of the corresponding mRNAs had occurred. The transmission of these activities to fresh cells was consistent with replication and packaging of the minigenomes into infectious virus particles. This is the first demonstration that bicistronic constructs can be used

to examine the regulatory functions of the junctional elements of HPIV3.

A. Junctional Elements

The junctional elements are believed to contain the *cis*-acting sequences necessary and sufficient for termination and reinitiation of the polymerase during transcription. The second major objective of this work was to examine the specific roles of these sequences during HPIV3 transcription.

1. Gene End Sequence

Deletion of the gene end sequence from the junction region (pPIV-CLΔGE; pPIV-LCΔGE) greatly inhibited expression of the upstream gene and abolished downstream gene expression (Figure 8). The reduction in upstream gene expression may result from polymerase readthrough or improper transcription termination. The reduction in downstream gene expression is also consistent with these two possibilities. These observations support the hypothesis that the gene end contains signals for transcription termination, and they also suggest that correct termination is required for reinitiation of transcription. For example, the polymerase may need to terminate at a precise position in order to "see" the downstream initiation site. If the polymerase is unable to terminate correctly, it may read through the junction. The

M gene end sequence, which contains eight extra nucleotides, appears to result in increased readthrough at this junction. The minigenome transcribed from pPIV-CL-M/F resulted in enhanced CAT expression; however, the length of this genome analog was a multiple of 6 nt, so interpretation of these results is difficult. Readthrough transcripts are not believed to be polyadenylated (Gupta and Kingsbury, 1985) which may result in unstable or non-functional messenger RNAs (Sachs, 1993).

The importance of the poly U stretch within the gene end sequence has also been demonstrated. No reduction in the length of this sequence was tolerated (Figure 10). The poly U stretch is believed to be the polyadenylation signal and template for paramyxoviruses (Prigden and Kingsbury, 1972; Schubert and Lazzarini, 1981; Hsu *et al.*, 1985). Premature termination and polymerase readthrough can only be distinguished by RNA analysis.

2. Gene Start Sequence

Mutations made to the gene start sequence effectively abolished expression of that gene (Figure 8). This observation is consistent with the transcription initiation site being located within these sequences. HPIV3 mRNAs have been shown to initiate at the 5′ nucleotide of the gene start sequence (Spriggs and Collins, 1986; Côté *et al.*, 1987). Because the expression of the upstream gene was enhanced when

downstream initiation was prevented, it is possible that the gene start sequence also influences transcription of the preceding gene.

Enhanced expression upstream upon removal of the gene start sequence may also be related to polymerase availability or 'bottle-neck' alleviation. If the polymerase cannot translocate properly to the downstream gene, it may dissociate from the template and become available to reinitiate at the 3' terminal promoter, increasing the number of transcripts produced for the upstream gene. This hypothesis assumes that the polymerase of nonsegmented negative stranded RNA viruses is limiting during infection, and the polymerase has been shown to be present in limiting amounts for VSV (Meier *et al.*, 1987). The gene junctions have also been described as bottle-necks due to processes that are slow relative to transcription itself, such as polyadenylation, termination, initiation and capping (Iverson and Rose, 1981). Thus, it is also possible that removing a sequence that causes the polymerase complex to pause may allow a greater number of polymerase molecules to finish transcribing the upstream gene in a given time period.

3. Intergenic Trinucleotide

The intergenic trinucleotide is nontranscribed, and has been suggested to act as part of the transcription initiation or termination signals (Hsu *et al.*, 1985; Re *et al.*, 1985).

The intergenic sequence for a number of paramyxoviruses is highly conserved; the consensus for measles virus, Sendai virus and HPIV3 is 3'-GAA. Because of its high degree of conservation, it was not surprising that most mutations made to this sequence were detrimental to gene expression. Decreasing the length of the intergenic sequence resulted in decreased CAT expression when the CAT gene was upstream or downstream of the junction (Figure 9). Altering the sequence of the trinucleotide resulted in decreased CAT expression when CAT was the upstream gene but normal levels of expression of the CAT gene when it was in the downstream position. These data indicate that correct transcription requires an intact intergenic sequence. One interpretation is that the intergenic sequence is important for transcription termination, while the length of the intergenic element has a role in transcription reinitiation.

Alterations made to the intergenic sequence of HPIV3 that kept the length constant resulted in decreased upstream CAT expression and normal levels of expression when the CAT gene was in the downstream position. Thus it seems that HPIV3 has less flexibility in the sequence of its intergenic element than do other paramyxoviruses such as RSV, NDV and mumps virus. The set of intergenic sequence mutants is the only example in our set of constructs of mutant minigenomes that resulted in expression of the downstream gene in the absence of expression of the upstream gene. If the

intergenic sequence is indeed part of the termination signals, then it is possible that an altered intergenic sequence results in improper termination or polyadenylation. The gene end may still be the major signal for transcription termination, but this signal may require presentation in the appropriate context for the viral polymerase to dissociate from the template. Analysis of Sendai DI strain 7a transcription has shown that the viral polymerase cannot terminate unless it encounters specific nucleotides downstream of the polyadenylation template (Hsu *et al.*, 1985). The intergenic sequence may also specifically direct polyadenylation via stuttering at the upstream U stretch. As mentioned, unpolyadenylated transcripts may be of poor quality. Because the next gene is still three nucleotides away, the polymerase can still reinitiate properly at the downstream gene.

An examination of Sendai virus internal deletion DI genomes has shown that the polymerase always terminates immediately before a purine trinucleotide (Re *et al.*, 1985). If termination requires a purine trinucleotide for termination, it would be predicted that a purine trinucleotide be present between the L gene and the trailer sequence. A similar sequence does exist for HPIV3 (3'-GUA), although U is not a purine. With the exception of minigenomes transcribed from pPIV-CL-IgGUA and pPIV-LC-IgGUA, the mutations made to the intergenic trinucleotide consisted

of various combinations of purine nucleotides. A set of mutant minigenomes with pyrimidine nucleotides in the intergenic position will help define the importance of the specific sequence of this region.

It appears that the length of the intergenic sequence itself is an important factor in transcription of several paramyxoviruses. Sendai virus and measles virus both have intergenic regions of three nucleotides; however, the length of the intergenic sequences of RSV varies from 2 to 52 nt (Collins *et al.*, 1986). It has been suggested that variable intergenic sequences provide a means of attenuation in viruses such as RSV and SV5. Perhaps for HPIV3, if the next gene is more than three nucleotides away, the polymerase cannot translocate properly to the next gene. Doubling the length of the intergenic element addresses this possibility. In minigenomes with duplicated intergenic elements, the gene end sequence and the gene start sequence are still in the same position relative to the intergenic sequence. However, these two elements are more separated from each other. Cells transfected with RNA transcribed from pPIV-LC+GAA did not express the CAT gene, while the minigenome transcribed from pPIV-CL+GAA resulted in increased CAT gene expression (Figure 6). If one assumes that the polymerase does not reinitiate at the downstream gene, upstream enhancement may result from polymerase availability or bottle-neck alleviation, as described above.

A minigenome was also constructed to address the question of internal polymerase initiation. Deleting the GAA from the leader region prevented transcription of both downstream genes. The 3'-GAAA(A) between the leader and N gene of VSV has been suggested to interact with the AU-rich leader sequence to terminate the polymerase before reinitiation at the NP gene (Keene *et al.*, 1980). For Sendai virus, the putative initiation sequences have been proposed to signal termination in conjunction with the purine trinucleotide (GAA) at the leader, where no other termination signals exist (Re *et al.*, 1985). The absence of the GAA in the leader sequence may not directly prevent initiation at the NP gene, but may result in nonfunctional, readthrough leader-NP polytranscripts. Leader-NP readthrough products detected in *in vitro* systems have been speculated to be abortive attempts at replication (Schubert and Lazzarini, 1981).

More analysis is needed to understand the specific coordination exhibited between the gene start, intergenic and gene end sequences.

B. Serial Passage

The ability to pass culture supernatants to fresh cells and detect CAT expression confirms transmissibility. In contrast to earlier reports (Dimock and Collins, 1993),

several outcomes were observed upon serial passage. The most frequent outcome of serial passage of the minigenome transcribed from pPIV-CL-NP/P was amplification of gene expression (Figure 11A). This observation may reflect competition and interference with helper virus, and is similar to the action of DI particles. CAT expression was occasionally seen to taper off (Figure 11B). This observation could be explained if the initial transfection was inefficient, and minigenomes were outcompeted by helper virus. In one case, CAT activity increased sharply after one of the passages. It is possible that a mutation had occurred in the minigenome sequence making transcription or replication more efficient. Viral RNA-dependant RNA polymerases introduce mutations at a high frequency. Perhaps a mutation occurred that converted the length of the minigenome to a multiple of six. The addition of 2 nt would make the length of the transcribed minigenome of pPIV-CL-NP/P divisible by six.

C. RNA Analyses

In order to confirm reporter results, RNA analyses were performed. It was expected that these studies would help explain some of the observations and distinguish between polymerase readthrough, improper polyadenylation, and changes in the amounts of mRNAs transcribed from the synthetic

minigenomes. Northern blotting and RNAse protection were performed to examine mRNA directly. Northern blotting, though it has the advantage of allowing visualization of the sizes of the individual RNA species, is considerably less sensitive than CAT expression (Calain *et al.*, 1992). RNAse protection is a better method for quantitation and is more sensitive than Northern blotting. While the data suggest that CAT specific mRNA species were detected, signals were generally low, amidst a high level of background hybridization (Figures 12-14). Troubleshooting with the system points to the initial transfection as being the inefficient step, though it is also possible that CAT specific mRNAs are unstable.

If the transfection efficiency can be improved, by altering the transfection agent or changing the amount or quality of input RNA, then serial passage of virus particles containing the minigenomes may be used to amplify the level of RNA. The level of amplification has been shown to be greater than 1000 fold after 5 passes (Dimock and Collins, 1993). Thus, all minigenomes could be passed several times before harvesting their RNA. As well, isolation of polyadenylated RNA will help reduce nonspecific hybridization to ribosomal RNA.

It has been observed for Sendai virus, which has been rescued using helper-dependent and independent techniques, that vaccinia virus driven rescue systems have an enhanced

ability to support replication compared to helper virus dependent systems (Calain *et al.*, 1992). Replication of VSV DIs using a vaccinia based rescue system is so efficient that "levels of synthesis after 18-24 hrs equal that of 28 and 18S rRNA" (Pattnaik *et al.*, 1992). Thus, a helper-independent rescue system may be more efficient for rescuing HPIV3 minigenomes. Such a system is being developed in our laboratory and will be used to look at the quality and quantity of mRNAs produced. As well, the presence of readthrough RNAs should be more easily detected. It should be noted that helper-independent rescue systems are not always the most efficient rescue techniques. Measles virus, for example, can be more efficiently rescued using a helper-dependent system (Kalin *et al.*, 1994). Thus, it remains to be seen which system will result in more efficient rescue for HPIV3.

D. The Rule of Six

The final aim of this project was to assess whether or not the `Rule of 6', proposed for negative stranded RNA viruses (Calain and Roux, 1992), applies to HPIV3. This rule states that the overall length of a viral RNA or RNA analog must be a multiple of 6 nt for efficient rescue. The `Rule of Six' was proposed for Sendai virus DI genomes and, therefore, addressed replication. The bicistronic rescue

system we have developed is ideal for studies of this kind. As each construct contains two reporter genes, the luc coding region could be manipulated without altering potential *cis*-acting sequences involved in transcription or replication. We observed that creating constructs that conform to the `Rule of Six' confers an advantage that results in increased reporter activity. Synthetic genome analogs that were multiples of six generally resulted in 5-10 fold greater levels of gene expression (Figure 12), although they could not compensate for mutations made in other *cis*-acting sequences that were detrimental to gene expression. Other work in this laboratory with monocistronic constructs has also shown that minigenomes that are multiples of 6 nt in length can be rescued more efficiently (K. Dimock, unpublished observations).

Looking simply at gene activity cannot distinguish between replicative or transcriptive advantages. However, it was seen that the Sendai DI particles that were multiples of six had increased levels of replication (Calain and Roux, 1992). The original studies into this phenomenon ruled out incorrect encapsidation as a reason for lack of replication in non-Rule of Six DIs, as RNAs were found in nucleocapsid structures banding on CsCl gradients (Calain and Roux, 1992). It was suggested that, as each NP molecule specifically binds 6 nucleotides, any extra nucleotides at the genomic termini would be available for RNAse digestion. Interestingly, the

pSend-CAT construct rescued by Park and colleagues (1991) in their Sendai system was a multiple of 6. As well, the lengths of the pSDI-CAT rabies analog (Conzelmann and Schnell, 1994), measles constructs that can be rescued (Kalin *et al.*, 1994), and pHPIV3-CAT (De and Banerjee, 1993) are all evenly divisible by 6. However, the suggested stoichiometry for VSV is 9 nt per N protein, and there seems to be no strict nt:N ratio for efficient rescue of VSV minigenomes (Pattnaik *et al.*, 1995). The RSV system also does not seem affected by this rule (P.L. Collins, personal communication).

It seems that minigenome replication in the HPIV3 rescue system is able to occur despite the fact that the length of the RNA is not a multiple of six. Indeed, the length of the HPIV3 genome is itself not divisible by 6. However, when the overall length of a transcribed minigenome is evenly divisible by 6 nt, rescue appears more efficient. This observation will have significance for others using this rescue system, as RNA species and reporter activity for analogs that have lengths that are multiples of six should be more easily detected. RNA analysis will help confirm that the `Rule of Six' confers a replicative advantage to HPIV3 minigenome RNA.

E. Prospects

Future work on this project should focus on determining the precise conditions for consistently efficient rescue. The initial transfection step needs to be carefully optimized. Then, RNA analyses specifically will help understand the mechanisms behind the changes in reporter activities observed.

Understanding the regulation of transcription of the negative stranded viruses is fundamental to understanding the pathogenesis of these viruses. For example, mutations in the P/M intercistronic region of measles virus have been related to several cases of subacute sclerosing panencephalitis (Crowley et al., 1988). Rescue systems will allow the direct analysis of the effects of these mutations.

A major goal of this laboratory is the expression of HPIV3 itself from a cloned DNA. The ability to produce infectious virus from cDNA will allow more specific analyses of the genes and regulatory sequences of the negative strand viruses. A possible end goal of such analyses is to create recombinant, attenuated viruses for vaccine use. Previous vaccine strategies for measles and RSV used inactivated virus; however, these vaccines resulted in exaggerated responses to natural infections (Subbarao and Murphy, 1992). Collins *et al.* (1993) have reported rescue of a RSV analog that is 49.3% of full length. Rescue of a full length genome

analog has recently been achieved for rabies virus (Schnell *et al.*, 1994).

Rescue systems also have specific applications, such as the expression of foreign genes. Preliminary work to this end has been reported for influenza A virus (Parvin *et al.*, 1994; Garcia-Sastre *et al.*, 1994). The ultimate goal of such studies is to use viral genome analogs as viral vectors in gene therapy. HPIV3 and other respiratory viruses may have specific applicability to introducing genes into the respiratory tract for treating such illnesses as cystic fibrosis.

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VII. APPENDIX Oligonucleotide Sequences

Primers used for sequencing

3061	5'-TTT ACG ATG CCA TTG GGA-3'	CAT + sense
3062	5'-CAT GTC GGC AGA ATG CTT-3'	CAT - sense
4492	5'-GCA AGA AAA ATC AGA GAG-3'	luc - sense
4516	5'-TTG CTC TCC AGC GGT TCC ATC-3'	luc + sense

Oligonucleotide pairs used for cloning HPIV3 junctional sequences

4528 5'-ATA AAT AAG AAA AAC TTA GGA TTA AAG A-3'
4529 5'-GAT CTC TTT AAT CCT AAG TTT TTC TTA TTT ATG C-3'

4530 5'-ATA ATT ATA AAA AAC TTA GGA GTA AAG A-3'
4531 5'-GAT CTC TTT ACT CCT AAG TTT TTT ATA ATT ATG C-3'

4532 5'-ATA AAT ATA AAA AAC TTA GGA GCA AAG A-3'
4533 5'-GAT CTC TTT GCT CCT AAG TTT TTT ATA TTT ATG C-3'

4534 5'-ATA AAT AAG AAA AAA GGA TTA AAG A-3'
4535 5'-GAT CTC TTT AAT CCT TTT TTC TTA TTT ATG C-3'

4536 5'-ATA AAT AAG AGA TAA TCA AAA ACT TAG GAT TAA AGA-3'
4537 5'-GAT CTC TTT AAT CCT AAG TTT TTG ATT ATC TCT TAT TTA TGC-3'

4538 5'-ATA AAT AAG AAA AAC TTA GGA CAA AAG A-3'
4539 5'-GAT CTC TTT TGT CCT AAG TTT TTC TTA TTT ATG C-3'

4608 5'-ATA AAT AAG AAA AAC TTA-3'
4609 5'-GAT CTA AGT TTT TCT TAT TTA TGC-3'

4610 5'-ATC TTA GGA TTA AAG A-3'
4611 5'-GAT CTC TTT AAT CCT AAG ATG C-3'

4612 5'-ATA AAT AAG AAA AAC TTC TTA GGA TTA AAG A-3'
4613 5'-GAT CTC TTT AAT CCT AAG AAG TTT TTC TTA TTT ATG C-3'

Primers used for site-directed mutagenesis of NP/P junction

4597 5'-TTT AAA TTA AAA TTA AAT TAT TAA AGA CAT TG-3'

5545 5'-GCA TAA ATA AGA AAA ACA TAG GAT TAA AGA GAT C-3'

5546 5'-GCA TAA ATA AGA AAA ACC TAG GAT TAA AGA GAT C-3'

5547 5'-GCA TAA ATA AGA AAA ATT TAG GAT TAA AGA GAT C-3'

5548 5'-GCA TAA ATA AGA AAA ACT CAG GAT TAA AGA GAT C-3'

5549 5'-GCA TAA ATA AGA AAA AAC TTA GGA TTA AAG AGA TC-3'

5550 5'-GCA TAA ATA AGA AAA CTT AGG ATT AAA GAG ATC-3'

5551 5'-GCA TAA ATA AGA AAC TTA GGA TTA AAG AGA TC-3'

5552 5'-GCA TAA ATA AGA ACT TAG GAT TAA AGA GAT C-3'

5553 5'-GCA TAA ATA AGA CTT AGG ATT AAA GAG ATC-3'

5554 5'-GCA TAA ATA AGA AAA ACT AGG ATT AAA GAG ATC-3'

5555 5'-GCA TAA ATA AGA AAA ATT AGG ATT AAA GAG ATC-3'

5556 5'-GCA TAA ATA AGA AAA ATA GGA TTA AAG AGA TC-3'

5557 5'-GCA TAA ATA AGA AAA ACA GGA TTA AAG AGA TC-3'

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